TRANSCRIPTIONAL REGULATION OF LIPID HOMEOSTASIS IN *DROSOPHILA*

by

Matthew H. Sieber

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STATEMENT OF DISSERTATION APPROVAL

The dissertation of **Matthew H. Sieber**

has been approved by the following supervisory committee members:

Carl S. Thummel	, Chair	**9/8/2011** Date Approved
Charles Murtaugh	, Member	**9/8/2011** Date Approved
Gillian Stanfield	, Member	**9/8/2011** Date Approved
Jared Rutter	, Member	**9/8/2011** Date Approved
Mark M. Metzstein	, Member	**9/8/2011** Date Approved

and by **Lynn Jorde** , Chair of

the Department of **Human Genetics**

and by Charles A. Wight, Dean of The Graduate School.

ABSTRACT

The ability to sense metabolic status and coordinately regulate specific aspects of metabolism is central to human health and disease. The second chapter of this dissertation defines a role for the *Drosophila* nuclear receptor, *DHR96*, as a key regulator that coordinates triacylglycerol (TAG) and cholesterol homeostasis. Studies presented in this dissertation show that DHR96 maintains whole animal TAG levels by promoting the breakdown of dietary TAG through regulation of the intestinal lipase Magro. Previous studies have shown that DHR96 binds cholesterol in its ligand-binding domain and regulates the transcriptional response to dietary cholesterol. The third chapter of this dissertation describes how, in conjunction with its role in promoting dietary TAG digestion, DHR96 functions through *magro* to prevent the accumulation of excess sterols in the fly. Magro exerts this role in cholesterol homeostasis through its cholesterol esterase activity, cleaving stored cholesterol esters to promote sterol excretion. Taken together, these studies define a key role for DHR96 in coordinating dietary TAG digestion and maintaining cholesterol homeostasis through its regulation of *magro*. Observations made during my studies of *Drosophila* lipid metabolism suggested that the metabolic state of the progeny is dependent on the health and nutritional status of their parents. The fourth chapter of this dissertation shows that temporary dietary restriction during the parental

generation influences TAG and glycogen levels in the resulting progeny, as well as specific changes in metabolic gene expression. This work also defines roles for both DHR96 and HP1 as key regulators of these transgenerational effects on metabolism, suggesting that transcriptional regulation and chromatin are a central part of this pathway. Overall, these studies establish a new genetic system and framework for detailed molecular characterization of the mechanisms that facilitate the transgenerational regulation of metabolism, including an investigation of nuclear receptor and chromatin factors in this process.

This dissertation is dedicated to my sister Melissa Sieber
and my mother Terry Sieber.
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I would first like to express my sincere gratitude to my advisor, Carl Thummel. From the first day I joined the lab, it became apparent that few people care for, or support, the people in their lab more than Carl. While he will challenge you and push you, he does so to make sure that you can reach your full potential. As a result of Carl's extraordinary ability as a mentor, he has taught me how to truly be a scientist, not just an experimentalist. Carl has shown me the correct way to put together a story, present it in public, and write it for publication, all essential skills for a successful career in science. For these reasons and many others I can honestly say that there are few people, let alone scientists, that I respect more than Carl. I'm very lucky for having the opportunity to work with him over the past few years and I feel that joining his lab was one of the best decisions that I have ever made.

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CHAPTER 1

INTRODUCTION

Triacylglycerol (TAG) and cholesterol homeostasis is maintained by balancing dietary lipid uptake and synthesis with lipid catabolism and excretion (Ikonen, 2008; Lusis and Pajukanta, 2008). Under normal feeding conditions, TAG and cholesterol are either synthesized or absorbed from the diet. These lipids can then be packaged in lipoprotein particles that are released into the circulatory system as a means to distribute them throughout the body. These trafficked lipids are either utilized by cells for the production of signaling molecules, membrane components, or deposited in storage tissues such as adipose tissue or the liver. When needed by cells, stored TAG and cholesterol esters can be broken down and utilized for energy production, or excreted to remove excess lipids.

Disruptions in lipid regulatory mechanisms have been implicated in a number of human diseases ranging from neurological disorders, such as Niemann-Pick Type C disease, to other more systemic conditions such as diabetes, obesity, and coronary heart disease. As a result, there has been a concerted effort in model system research to identify the regulatory factors that maintain lipid homeostasis. These studies have identified *SREBP* and *LXR* as key transcription factors that control lipid synthesis and cholesterol excretion (Briggs et al., 1993; Hua et al., 1993; Janowski et al., 1996; Peet et al., 1998; Wang et al., 1993; Yokoyama et al., 1993). Despite these studies, little is known about how the target genes of these transcription factors coordinate the complex physiological pathways that maintain lipid homeostasis.

SREBP-mediated control of TAG and cholesterol homeostasis

One of the best characterized factors that coordinates cholesterol and TAG metabolism is the SREBP signaling pathway. *SREBP-1c* and *SREBP2* encode helix-loop-helix leucine zipper transcription factors that localize to the endoplasmic reticulum (Hua et al., 1993; Yokoyama et al., 1993). When cellular cholesterol levels decrease, SREBPs are activated by a series of proteolytic cleavage events that release the N-terminal domain from the ER. The cleaved protein enters the nucleus where it activates the expression of target genes involved in cholesterol and triglyceride metabolism (Wang et al., 1994). *SREBP-1c* controls the fatty acid and TAG synthesis branch of the SREBP signaling pathway by regulating the expression of key enzymes like Acyl-CoA synthase (ACC) and fatty acid synthase (FAS) (Figure 1.1 and 1.2) (Bennett et al., 1995; Briggs et al., 1993; Magana et al., 1997; Wang et al., 1993). In contrast, SREBP2 controls the cholesterol synthesis arm of the pathway through direct regulation of HMG-CoA reductase and several other enzymes in the cholesterol synthesis pathway (Figure 1.2) (Vallett et al., 1996). SREBP activation promotes *de novo* TAG and cholesterol synthesis as well as increased lipid uptake by the cell, providing a means of coordinating cellular lipid status with TAG and cholesterol homeostasis (Osborne, 2000; Vallett et al., 1996). Despite detailed studies of this pathway, much remains to be learned about the factors that function upstream of SREBP to regulate its expression and proteolytic activation.

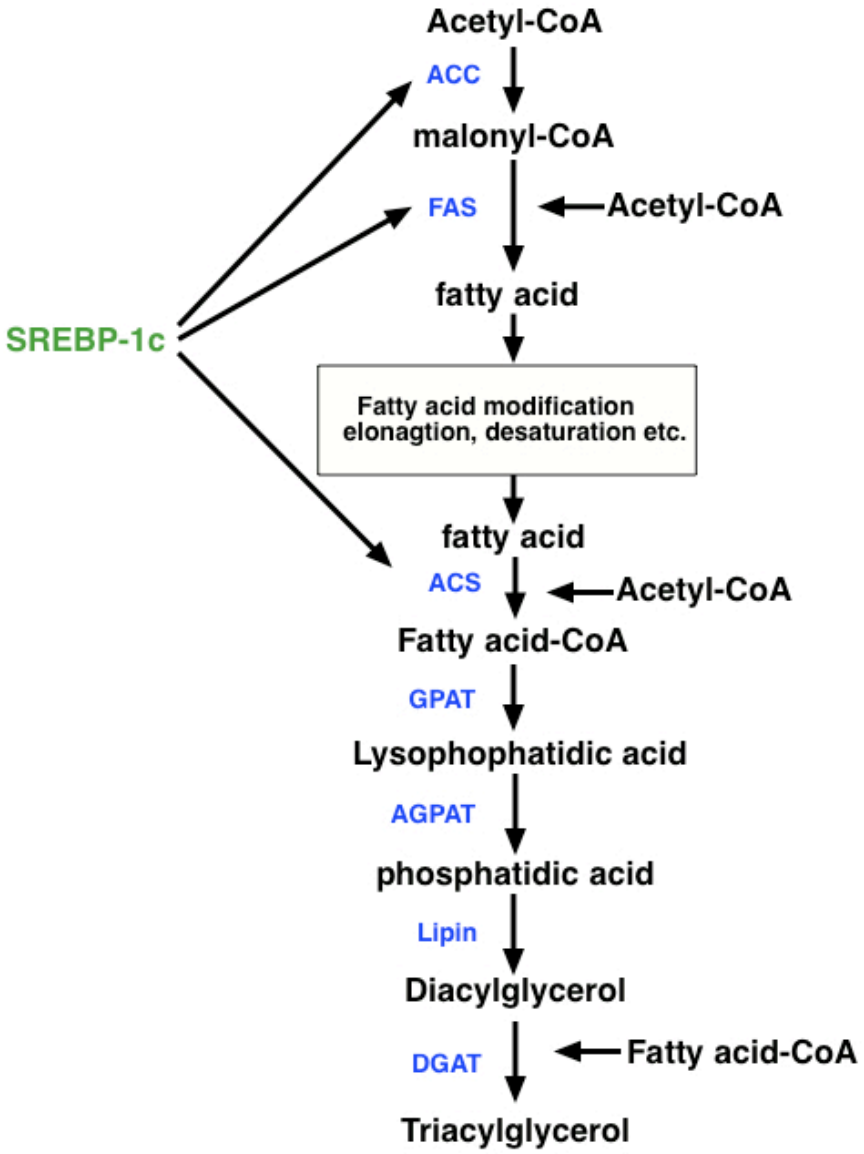

Figure 1.1 triglyceride biosynthetic pathway

This figure depicts the biosynthetic enzymes (blue) that convert acetyl-CoA, through a series of intermediates (black) into triacylglycerol.

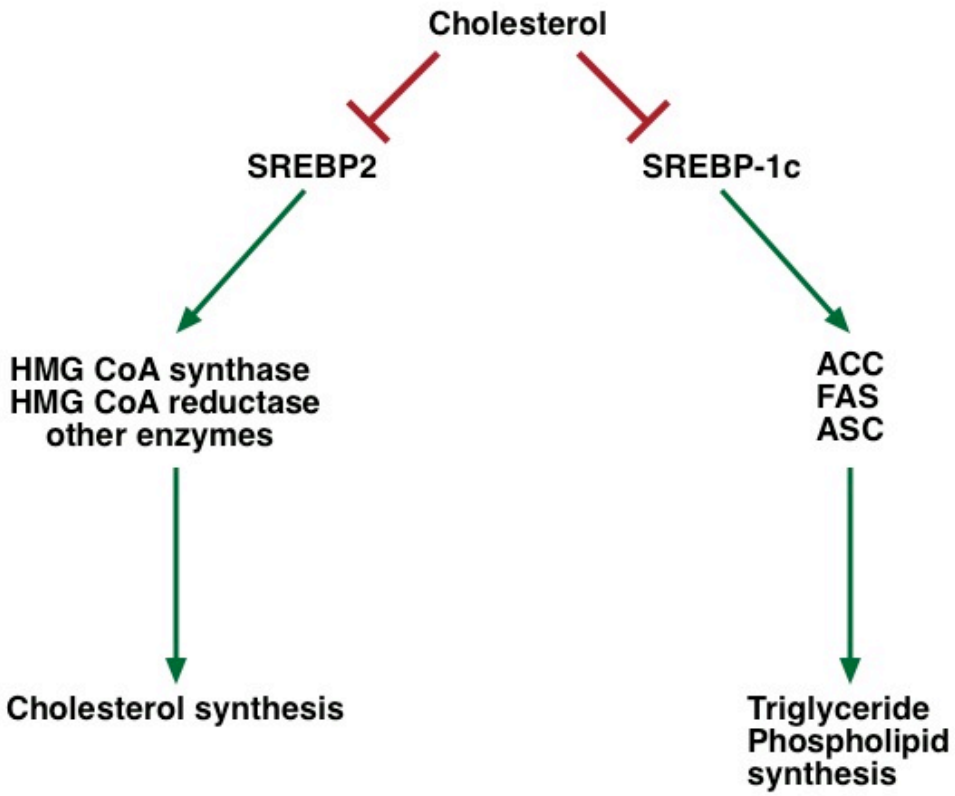

Figure 1.2 The SREBP signaling pathways

Under conditions of low cellular cholesterol levels, both SREBP2 and SREBP-1c are cleaved and enter the nucleus to activate the expression of genes involved in cholesterol and fatty acid/TAG synthesis, respectively.

Regulation of TAG and cholesterol homeostasis by the LXR subclass of nuclear receptors

Nuclear receptors (NRs) are ligand-regulated transcription factors that play central roles in multiple aspects of lipid metabolism. Many NRs bind small lipophilic compounds, such as fatty acids, sterols, and bile acids, and direct specific changes in gene expression. One example of this is LXRα (NR1H3), which binds oxysterols in its ligand binding domain and regulates both TAG and cholesterol metabolism (Janowski et al., 1996; Kalaany and Mangelsdorf, 2006). Mutants lacking *LXRα* display elevated levels of circulating and stored cholesterol in the liver. LXRα maintains cholesterol homeostasis through direct regulation of bile salt production through the CYP7A2 bile acid biosynthetic enzyme, and bile excretion through the *ABCG5/ABCG8* bile acid transporters (Figure 1.3) (Repa et al., 2002). Genetic studies have shown that *LXRαβ* double mutant mice, while displaying normal body weight and body fat, are resistant to diet-induced obesity, also implicating a role for this receptor in TAG metabolism. LXRs function in the liver to maintain TAG homeostasis by controlling fat synthesis and uptake, in part, through direct transcriptional regulation of the key lipogenic transcription factor *SREBP-1c* (Figure 1.3) (Schultz et al., 2000).

LXR activity is thus central to both TAG and cholesterol homeostasis, although much remains to be learned about the roles of specific LXR target genes in mediating these key metabolic functions.

In addition to its role in the liver, recent work has identified an important role for LXRs in the regulation of cholesterol excretion in the intestine. LXR activation in this tissue results in a dramatic increase in fecal sterol excretion that correlates with increased

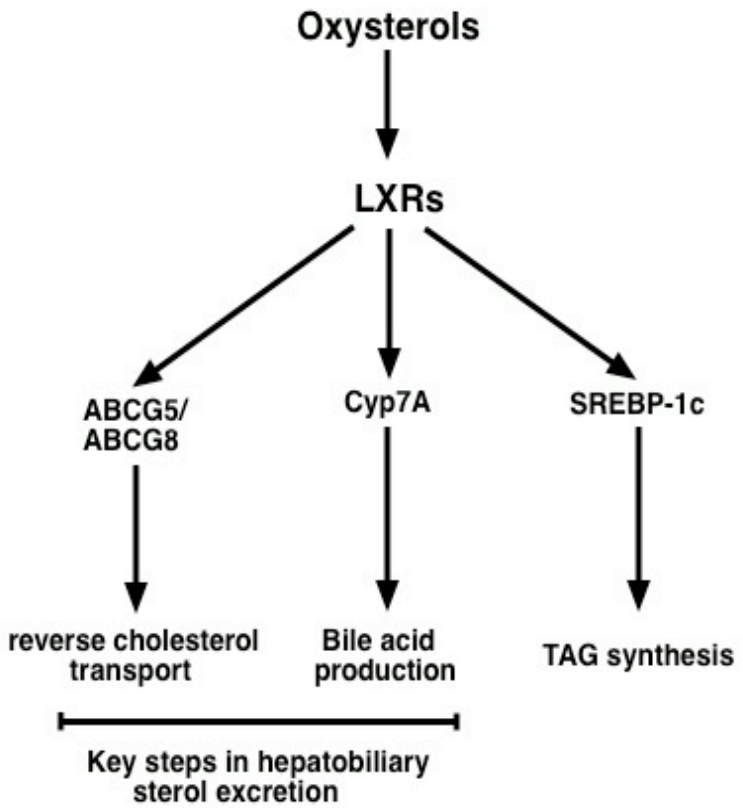

Figure 1.3 LXRs coordinate cholesterol and TAG homeostasis

In response to increasing oxysterol levels, LXRs are activated in the liver, inducing the expression of genes involved in hepatobiliary cholesterol excretion and liver fatty acid/ TAG synthesis.

expression of the *ABCG5/ABCG8* bile acid transporter (Lo Sasso et al., 2010; van der Veen et al., 2009). This observation suggests that LXR promotes reverse cholesterol transport in this tissue, which represents one of the best-characterized mechanisms for eliminating excess cholesterol from the body. Reverse cholesterol transport involves HDL-mediated transport of cholesterol from peripheral tissues to the hepatobiliary tract, leading to the removal of excess sterol by biliary excretion from the body. LXR

activation of fecal sterol excretion, however, proceeds normally in *abcb4* mutant mice, which have severely impaired biliary cholesterol secretion (Kruit et al., 2005). Similarly, *abcg5*/*abcg8* double mutants, which have highly reduced biliary cholesterol excretion, have relatively normal levels of fecal sterols (Yu et al., 2002). These and other studies have challenged the importance of reverse cholesterol transport for cholesterol excretion and have led to the proposal that the intestine may play a more direct role in this process (van der Velde et al., 2010; van der Velde et al., 2007). Indeed, intestinal perfusion studies in mice have demonstrated that up to 60% of fecal sterols can be excreted directly from the proximal intestine (van der Velde et al., 2007). Taken together, these studies are shifting the focus of cholesterol efflux toward the intestine and implicate a central role for LXR in regulating this poorly understood pathway.

LipA-mediated TAG and cholesterol homeostasis

While there have been numerous studies of the transcriptional regulation of lipid metabolism, some enzymes can also play a central role in coordinating cholesterol and TAG homeostasis. For example, the *LipA/LAL* gene, which encodes an acid lipase, is mutated in patients suffering from cholesterol ester storage disease (CESD) and Wolman's disease (Burke and Schubert, 1972; Goldstein et al., 1975; Pisciotta et al., 2009). These patients exhibit digestive difficulties and have significantly reduced deposits of white adipose tissue, while at the same time displaying increased TAG and cholesterol accumulation in the liver and spleen (Crocker et al., 1965; Kyriakides et al., 1970; Ozsoylu et al., 1977; Pisciotta et al., 2009). Similar effects have been seen in genetic studies of *LipA* null mutant mice (Du et al., 1998; Du et al., 2001). Biochemical

studies of LipA have demonstrated that it acts as a dual function enzyme with both cholesterol esterase and TAG lipase activity (Ameis et al., 1994; Goldstein et al., 1975), suggesting that it may directly impact both TAG and cholesterol metabolism. Studies of macrophage cholesterol efflux have shown that this enzyme breaks down stored cholesterol esters as a means of promoting ABCA1-mediated cholesterol efflux (Ouimet et al., 2011). Roles for LipA in TAG homeostasis, however, remain to be determined.

Drosophila as a simple system for studying TAG metabolism

The complex nature of metabolic disorders has made it difficult to determine the physiological mechanisms that control lipid homeostasis. Given the advanced genetic tools available, and the fact that many metabolic regulatory pathways are conserved from flies to humans, *Drosophila* provides a simple system for the physiological and molecular characterization of the mechanisms that maintain metabolic homeostasis (Baker and Thummel, 2007; Kuhnlein, 2010). This was initially seen in studies of the *Drosophila adipose* gene, which was identified in genetic studies of a naturally-occurring obese fly stain. Mutants lacking *adipose* function exhibit a significant increase in whole animal TAG levels (Doane, 1960). Subsequent studies have shown that *adipose* acts in a dose dependent manner. It appears to antagonize TAG accumulation and TAG synthesis in both flies and mice, through interactions with histones and HDAC3 to modulate chromatin state (Suh et al., 2007). Mutations in *adipose* have also been associated with obesity in humans (Lai et al., 2009). This work highlights the use of *Drosophila* as an ideal system for the discovery of novel TAG regulatory mechanisms that are conserved through evolution, from flies to humans. Genetic studies of lipid metabolism in

Drosophila, however, have only been underway for a few years. Below I discuss some major advances in this area, outlining roles for key regulators such as SREBP, Seipin, and factors involved in lipid storage, breakdown, and transport.

Fatty acid synthesis metabolism in *Drosophila*, as in mammals, initiates with acetyl-CoA precursors through the activity of ACC and FAS (Figure1.1). Free fatty acids are then activated by acyl-CoA synthase and esterified to a glycerol backbone, producing acylglycerol lipids such as diacylglycerol (DAG) and TAG, the circulating and stored form of fat in the fly. Interestingly, SREBP has been identified as a key transcriptional regulator that controls the expression of all three enzymes in this lipid biosynthetic pathway in *Drosophila* (Figure 1.1). *SREBP* mutants arrest in the second larval instar and exhibit decreased levels of TAG and fatty acid synthesis (Kunte et al., 2006). Furthermore, SREBP activity is stimulated by fatty acids, such as palmitate, and appears to function in multiple key metabolic tissues such as the fat body, intestine, and the oenocytes, indicating a role in the nutrient-dependent control of lipid metabolism (Seegmiller et al., 2002).

Mutations in the Berardinelli-Seip Congenital Lipodystrophy (BSCL) gene, *BSCL2,* in mammals are associated with a severe form of lipodystrophy where patients display severely depleted peripheral white adipose tissue (WAT) as well as ectopic lipid accumulation in nonlipid storage tissues such as muscle (Magre et al., 2001). Subsequent genetic studies of *seipin*, the *Drosophila* ortholog of *BSCL2*, have shown that mutants lacking *seipin* function display an 80% decrease in whole animal acylglycerols with ectopic lipid accumulation in the salivary glands. These defects result from of a block in phosphatidic acid utilization in the mutants and suggest a role for *seipin* in the early steps

of TAG synthesis, although the precise mechanisms remain to be determined (Tian et al., 2011).

In mammals, lipid trafficking is facilitated by lipoprotein particles composed of various lipids (TAG, cholesterol, cholesterol esters, phospholipids) and apolipoproteins that function as carriers. *RfaBp*/*lipophorin (Lpp)* mediates this lipoprotein carrier function in *Drosophila*. Animals lacking *Lpp* function display defective lipid accumulation in a number of tissues, including the fat body, midgut, and imaginal discs (Eugster et al., 2007; Panakova et al., 2005). These studies also indicate that *Lpp* is required for long-range *Wnt* and *Hedgehog* signaling. Once in circulation these lipoprotein particles are bound and absorbed by peripheral tissues so that the lipids they carry can be stored and/or utilized. Recent work has identified *lpr1* and *lpr2* as key membrane receptors that mediate the uptake of lipophorin particles in the ovary and imaginal discs (Parra-Peralbo and Culi, 2011). No significant changes, however, were observed in fat body lipid accumulation. These data suggest that a major source of fat body lipids derives from *de novo* fat synthesis and not from dietary lipids, although the source of these lipids has yet to be determined.

Once TAG is synthesized and trafficked to storage tissues such as the fat body and intestine, it is deposited in lipid droplets. This process of lipid storage requires the opposing functions of the lipid droplet surface proteins, plin1 and plin2, which are members of the perilipin family of proteins. In mammals, perilipins facilitate TAG storage through interactions with two key TAG lipases, hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) (Brasaemle, 2007). Mutants lacking *plin1,* are obese, displaying a 2-fold increase in TAG levels and a significant increase in lipid

droplet size (Beller et al., 2010). In contrast, *plin2* mutants display a ~35% decrease in whole animal TAG, consistent with its conserved role in antagonizing fat body lipolysis (Fauny et al., 2005; Gronke et al., 2003). Taken together, these studies suggest that the opposing functions of *plin1* and *plin2* control a balance between TAG storage and mobilization that is required to maintain whole animal lipid homeostasis. The precise molecular mechanisms by which *plin1* and *plin2* modulate TAG levels, however, have yet to be determined.

Stored TAG can be mobilized from lipid droplets by the activity of fat body TAG lipases to facilitate trafficking and utilization by peripheral tissues. Genetic studies of the *Drosophila ATGL* homolog, *brummer,* have shown that these mutants are severely obese as a result of their inability to breakdown stored TAG in the fat body (Gronke et al., 2005). Furthermore, analysis of *plin2;bmm* double mutants has shown that these null mutations mutually suppress each other, displaying an intermediate TAG phenotype. These results suggest that these genes function through parallel mechanisms to maintain TAG homeostasis. In addition, while critical for fat body TAG lipolysis, *brummer* mutants still exhibit significant starvation-induced fat body lipolysis, suggesting that there may be multiple mechanisms for the breakdown of stored TAG (Gronke et al., 2005). Recent data have identified a parallel lipolytic mechanism regulated by adipokinetic hormone (AKH) signaling, a pathway similar to the glucagon signaling pathway in mammals, that functions in the early stages of starvation to promote TAG breakdown (Gronke et al., 2007; Lee and Park, 2004). In the absence of both *AKH* signaling and *brummer* function, starvation-induced TAG lipolysis is lost, indicating that

these two pathways provide the primary means of fat body lipolysis in the fly, similar to the functions of *HSL* and *ATGL* in mammals (Gronke et al., 2007; Schweiger et al., 2006).

The free fatty acids that derive from TAG lipolysis can be utilized for energy production through the process of mitochondrial fatty acid β-oxidation. In this process, fatty acids are broken down into acetyl-CoA that enters the TCA cycle, driving oxidative phosphorylation to produce ATP. While fatty acid β-oxidation functions constitutively at low levels, maximal ATP production through β-oxidation and oxidative phosphorylation requires the transcriptional induction of key rate-limiting enzymes such as *CPT1* (carnitine acyltransferases) by the nuclear receptor *HNF4* in *Drosophila* (Palanker et al., 2009). Under starvation conditions, free fatty acids are released and can be bound by the ligand-binding domain of HNF4, activating the receptor (Palanker et al., 2009; Wisely et al., 2002). Once activated, HNF4 stimulates a feed-forward pathway that induces the expression of fatty acid β-oxidation enzymes as well as TAG lipases, promoting fat breakdown and energy production (Palanker et al., 2009). While *HNF4* mutants display normal TAG and fatty acid levels under fed conditions, starved *HNF4* mutants exhibit higher levels of both TAG and fatty acids, consistent with a block in TAG mobilization and β-oxidation. These data demonstrate a role for *HNF4* in promoting maximal TAG oxidation during starvation and suggest that alternative mechanisms regulate lipid oxidation under fed conditions.

Although these studies provide a good foundation for understanding the regulation of lipid homeostasis in *Drosophila*, a number of areas require further research. For example, although SREBP exhibits a conserved role in controlling fatty acid and triglyceride synthesis through its regulation of ACC, ACS, and FAS expression, much is

still unknown about the global role of *SREBP* in the transcriptional control of lipid homeostasis, as well as the precise signals that facilitate its cleavage and activation in *Drosophila*. In addition, aspects of lipid metabolism such as the intracellular trafficking of lipids, lipophorin particle assembly, and the content, secretion, and absorption of lipoprotein particles by peripheral tissues, remain unclear. Furthermore, although overexpression of insulin-like proteins in *Drosophila* stimulates whole animal TAG accumulation, the mechanisms that underlie insulin-mediated inhibition of lipolysis have not been defined (Arquier et al., 2008; Carnie et al., 1979). Finally, surprisingly little is known about the transcriptional regulatory mechanisms that facilitate nutrient digestion and absorption in response to feeding.

Regulation of cholesterol homeostasis in *Drosophila*

Cholesterol metabolism begins with the synthesis of cholesterol from acetyl-CoA precursors through the mevalonate and steroid biosynthetic pathway. *Drosophila*, despite having a functional mevalonate pathway, are unable to synthesize cholesterol *de novo*. This is a result of the fly lacking several key enzymes downstream of farnesyl pyrophosphate synthase in the conversion of mevalonate into cholesterol (Vinci et al., 2008). As a result, *Drosophila* are complete cholesterol auxotrophs and must rely on the absorption and conversion of dietary sterols for survival (Sang, 1956). In mammals, cholesterol absorption is facilitated by the Niemann-Pick type C1-like protein NPC1L1. Recent work has shown that the fly *NPC1L1* ortholog, *NPC1b,* plays a similar role in the midgut to mediate the absorption of dietary sterols. Mutants lacking *NPC1b* die during the second larval instar as a result of defective sterol absorption (Voght et al., 2007).

NPC1b mutants, however, do not exhibit changes in whole animal cholesterol levels, suggesting that there are additional mechanisms for sterol uptake present in the fly, and that NPC1b may have additional uncharacterized functions that may account for the lethality of *NPC1b* mutants.

Bulk sterols are trafficked through the ER and Golgi to be stored in lipid droplets, trafficked to the membrane, or packaged for release into the circulatory system to be distributed throughout the body. The *NPC2* family of proteins have been implicated as key factors required for subcellular sterol trafficking in both flies and mammals. While mice encode a single *NPC2* gene in their genome, *Drosophila* have eight members of this family, *NPC2a-h.* Initial studies of the *NPC2* genes in the fly indicate that mutants lacking *NPC2a* or *NPC2b* die during larval development and exhibit neuronal defects (Huang et al., 2007). Furthermore, when stained with the sterol-specific compound filipin, *NPC2a* mutants exhibit a punctate subcellular pattern indicative of a defect in cholesterol trafficking (Huang et al., 2007). Double mutant analysis suggests that *NPC2a* and *NPC2b* function in a semiredundant manner to promote cholesterol trafficking and utilization within the cell. Furthermore, the lethal phenotype observed in these double mutants can be suppressed by feeding excess cholesterol or the insect steroid hormone 20-hydroxyecdysone, indicating that steroid hormone production is restricted by the amount of available cholesterol.

The *Drosophila* ortholog of mammalian NPC1, NPC1a*,* exhibits a conserved role in intracellular post-lysosomal trafficking of sterols. *NPC1a* mutants display defective cholesterol trafficking as well as larval lethality (Fluegel et al., 2006). Moreover, this lethal phase can be suppressed by feeding 20-hydroxyecdysone, suggesting that NPC1a

functions with NPC2 proteins to mediate intracellular lipid trafficking. While these data are consistent with roles in cholesterol trafficking, however, little is known about the precise functions of these proteins. For example, it would be interesting to determine if these proteins act through a hydrophobic hand-off mechanism, whereby NPC2 directly transfers cholesterol to membrane bound NPC1, as described in mammals (Wang et al., 2010).

In addition to this role in cholesterol trafficking, the *Drosophila* NPC1 ortholog, *NPC1a*, appears to act in a parallel pathway to prevent intestinal cholesterol absorption (Wang et al., 2010; Xie et al., 1999). *NPC1a* mutants display elevated levels of cholesterol absorption, although the cause of this phenotype is unknown (Fluegel et al., 2006). Thus, while *NPC1b* functions to facilitate cholesterol absorption, *NPC1a* antagonizes cholesterol absorption. This model is supported by genetic analysis, which shows that null mutations in these two genes result in mutual suppression of their respective cholesterol absorption defects, yielding an intermediate phenotype. This observation suggests that NPC1a and NPC1b act in parallel to control sterol absorption. No changes in total cholesterol levels, however, were observed in either *NPC1a* or *NPC1b* mutants, indicating that these genes may have additional roles in cholesterol utilization that may account for the lethal phenotype of the mutants (Voght et al., 2007). More studies are required to define the precise mechanisms by which these NPC family members control cholesterol absorption and trafficking.

A major advance in understanding the regulation of cholesterol metabolism in *Drosophila* arose from biochemical and genetic characterization of the LXR nuclear receptor homolog, DHR96. In response to high levels of dietary cholesterol, DHR96

binds cholesterol in its ligand binding domain, much like LXR. *DHR96* mutants are lean and exhibit elevated levels of dietary cholesterol (Bujold et al., 2010; Horner et al., 2009; Sieber and Thummel, 2009). Microarray studies have identified a number of target genes for this receptor, many of which are expressed in the intestine and are predicted to function in lipid metabolism. These include *CG8112*, which encodes a predicted acyl CoA: cholesterol acetyltransferase (ACAT), *CG6472*, which encodes a homolog of lipoprotein lipase (LPL), the NPC2 family orthologs *npc2b, npc2c, and npc2d,* as well as a *LipA* homolog *magro (CG5932)* (Bujold et al., 2010; Horner et al., 2009; Sieber and Thummel, 2009). Functional studies of one of these targets, NPC1b, demonstrated that its lack of repression in *DHR96* mutants could account for the elevated sterol levels seen in these animals when they are maintained on a high cholesterol diet (Horner et al., 2009). *DHR96* mutants, however, do not show an overt defect in cholesterol absorption, indicating that this receptor likely functions to maintain cholesterol levels through the regulation of multiple aspects of cholesterol homeostasis. In addition, further studies are required to define roles for DHR96 in the regulation of TAG metabolism.

Thesis summary

In this dissertation I present my studies of DHR96 and its direct target gene, the *LipA* homolog *magro (CG5932).* In Chapter 2, I describe how *Drosophila* DHR96 regulates whole animal TAG homeostasis through the gastric lipase activity of Magro. Mutants lacking *DHR96* are lean, starvation sensitive, and resistant to diet-induced obesity. This decrease in whole animal TAG levels is correlated with a dramatic reduction in *magro* expression in the intestine. Midgut-specific expression of *magro* is

sufficient to rescue the *DHR96* mutant lean phenotype, demonstrating that this lipase plays a key role in the DHR96 signaling pathway and identifying DHR96 as a novel nutrient-responsive transcriptional regulator of dietary lipid metabolism.

In Chapter 3, I expand my studies of *DHR96* and *magro* by examining their roles in coordinating TAG and cholesterol homeostasis. In addition to better defining the regulation of dietary TAG metabolism, this work identifies a novel role for *DHR96* and *magro* in preventing the accumulation of excess sterols. Animals lacking *DHR96* or *magro* function exhibit elevated levels of cholesterol. My data indicate that these defects are due, in part, to an inability to break down stored cholesterol esters in the intestine. Taken together, my studies describe a mechanism by which nuclear receptors can coordinate multiple aspects of lipid homeostasis and demonstrates that a single direct target of these receptors can exert distinct key functions in different regions of the intestine.

Finally, in Chapter 4, I describe a new direction for my research that arose from my studies of *DHR96* and *magro*. In the course of this work, I noticed that wild-type flies display significant changes in absolute levels of TAG between experiments, and that these changes were not always apparent in the *DHR96* mutant. A series of studies revealed that these changes in TAG levels were not due to nutritional effects on that generation of flies, but rather reflected the health and nutrition of their parents. As a result, I began investigating the impact of parental diet on the metabolic status of their progeny. When parental flies are transiently exposed to a nutrient poor diet, the resulting progeny show an ~2-fold increase in whole animal TAG levels. Furthermore, mutants lacking *DHR96* function are unable to mediate this transgenerational response to parental

diet, suggesting that this receptor plays a key role in this pathway. These transgenerational effects on metabolic status are likely mediated by epigenetic changes in chromatin state. This hypothesis is supported by studies of animals heterozygous for a mutation in *HP1*, which encodes a conserved chromosomal protein that is involved in heterochromatin formation. My data support the model that transgerational changes in chromatin state adjust the metabolic state of the progeny to provide them with a physiological advantage for survival in their nutritional environment.

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CHAPTER 2

THE DHR96 NUCLEAR RECEPTOR CONTROLS TRIACYLGLYCEROL HOMEOSTASIS IN *DROSOPHILA*

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Cell Metabolism
Article

The DHR96 Nuclear Receptor Controls Triacylglycerol Homeostasis in *Drosophila*

Matthew H. Sieber[1] and Carl S. Thummel[1,*]
[1]Department of Human Genetics, University of Utah School of Medicine, 15 North 2030 East, Room 2100, Salt Lake City, UT 84112-5330, USA
*Correspondence: carl.thummel@genetics.utah.edu
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SUMMARY

Triacylglycerol (TAG) homeostasis is an integral part of normal physiology and essential for proper energy metabolism. Here we show that the single *Drosophila* ortholog of the PXR and CAR nuclear receptors, DHR96, plays an essential role in TAG homeostasis. *DHR96* mutants are sensitive to starvation, have reduced levels of TAG in the fat body and midgut, and are resistant to diet-induced obesity, while *DHR96* overexpression leads to starvation resistance and increased TAG levels. We show that *DHR96* function is required in the midgut for the breakdown of dietary fat and that it exerts this effect through the *CG5932* gastric lipase, which is essential for TAG homeostasis. This study provides insights into the regulation of dietary fat metabolism in *Drosophila* and demonstrates that the regulation of lipid metabolism is an ancestral function of the PXR/CAR/DHR96 nuclear receptor subfamily.

INTRODUCTION

Fat metabolism is central to the process of energy homeostasis. When nutrients are abundant, dietary fat in the form of triacylglycerol (TAG) is broken down by gastric TAG lipases to release fatty acids. These fatty acids are absorbed by the intestine and used to resynthesize TAG in peripheral tissues. These TAG reserves can be accessed upon nutrient deprivation through the action of specific lipid droplet-associated lipases that release the fatty acids for energy production through mitochondrial fatty acid β-oxidation. Defects in these processes can lead to dramatic changes in TAG levels and a range of physiological disorders, including obesity, diabetes, and cardiovascular disease. The alarming rise in the prevalence of these disorders in human populations has focused attention on understanding the molecular mechanisms that coordinate dietary nutrient uptake with TAG homeostasis. As a result, many regulators of TAG metabolism have been identified, including SREBP, PPAR, and adiponectin. In spite of these advances, however, the molecular mechanisms that coordinate dietary fat uptake, synthesis, storage, and utilization remain poorly understood.

Nuclear receptors (NRs) are ligand-regulated transcription factors that play a central role in metabolic control. They are defined by a conserved zinc-finger DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD) that can impart multiple functions, including hormone binding, receptor dimerization, and transactivation. Many NRs are regulated by small lipophilic compounds that include dietary signals and metabolic intermediates, and exert their effects by directing global changes in gene expression that act to maintain metabolic homeostasis. This is exemplified by members of the mammalian PPAR, LXR, and FXR subfamilies, which play critical roles in adipogenesis, lipid metabolism, and cholesterol and bile acid homeostasis, respectively (Chawla et al., 2001; Sonoda et al., 2008).

In this study we use the fruit fly, *Drosophila melanogaster*, as a simple model system to characterize the NR subfamily represented by the Pregnane X Receptor (PXR, NR1I2), Constitutive Androstane Receptor (CAR, NR1I3), and Vitamin D Receptor (VDR, NR1I1) in mammals. Previous studies have defined central roles for these receptors in sensing xenobiotic compounds and directly regulating genes involved in detoxification (Timsit and Negishi, 2007; Willson and Kliewer, 2002). Initial studies showed that the single ancestral *Drosophila* ortholog of this NR subclass, DHR96, has similar functions (King-Jones et al., 2006). A *DHR96* null mutant displays increased sensitivity to the sedative effects of phenobarbital and the pesticide DDT as well as defects in the expression of phenobarbital-regulated genes. These studies, however, revealed other potential roles for the receptor—in particular, unexpected effects on the expression of genes that are predicted to regulate lipid and carbohydrate metabolism (King-Jones et al., 2006). This observation is in line with recent studies that have implicated roles for the mammalian PXR and CAR NRs in metabolic control (Moreau et al., 2008). The molecular mechanism by which they exert this effect, however, remains undefined.

Here we show that *DHR96* null mutants are sensitive to starvation and have reduced levels of TAG, while DHR96 overexpression leads to starvation resistance and elevated TAG levels. A series of studies using metabolic assays, diets, and the drug Orlistat revealed that *DHR96* mutants are defective in their ability to break down dietary lipid. This model was supported by microarray studies, which showed that many genes expressed in the midgut are misregulated in *DHR96* mutants, including highly reduced expression of the gastric lipase gene *CG5932*. We show that *CG5932* is required for proper whole-animal TAG levels, and that selective expression of *CG5932* in the midgut of *DHR96* mutants is sufficient to rescue their lean phenotype. Taken together, these data support a role for the PXR/CAR/DHR96 NR subclass in lipid metabolism and define *DHR96* as a key regulator of dietary TAG breakdown in the *Drosophila* midgut.

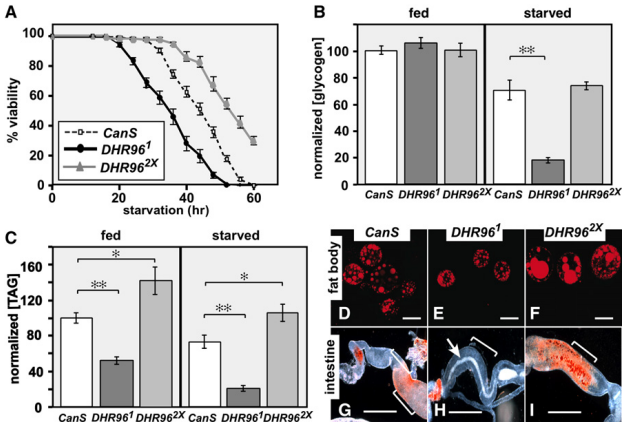

Figure 1. *DHR96* Mutants Are Sensitive to Starvation and Display Decreased Levels of TAG

(A) Mature adult male *Canton-S* (*CanS*) control flies, *DHR96*^{1} mutants, and the *DHR96*^{2X} overexpression strain were subjected to complete starvation, and the number of surviving animals was determined at 4 hr intervals. Results are presented as a percent of the total population, which consisted of 15 groups of 20 animals for each genotype. Similar results were obtained in a second independent experiment as well as from adult flies staged at 1–2 days after eclosion.

(B and C) Total glycogen (B) and TAG (C) levels were measured in *CanS* control flies, *DHR96*^{1} mutants, and the *DHR96*^{2X} overexpression strain under both fed conditions and after a 20 hr starvation. Glycogen and TAG levels were normalized for total protein and are presented as normalized to a wild-type level of 100%.

(D–F) Nile red staining of dissected fat body cells from 1- to 2-day-old adult male flies shows reduced cell size and lipid droplet size in *DHR96*^{1} mutants (E) and increased lipid droplet size in *DHR96*^{2X} animals (F), relative to *CanS* controls (D).

(G–I) Oil red O staining of dissected adult midguts shows reduced lipid accumulation in the *DHR96*^{1} mutant (H) and enlarged lipid droplets in the *DHR96*^{2X} overexpression strain (I), relative to *CanS* controls (G). The white brackets mark comparable regions of the midgut epithelium. The lumen of the midgut can be seen in the *DHR96*^{1} mutant (arrow). Scale bars are 75 μm (D–F) or 400 μm (G–I). Error bars represent ±SE, *p < 0.05 and **p < 0.001.

RESULTS

DHR96 Mutants Are Lean and Sensitive to Starvation

Previous studies have shown that *DHR96*^{1} null mutants, grown under normal conditions, are viable and fertile with no apparent developmental defects (King-Jones et al., 2006). To assess potential metabolic roles for *DHR96*, we examined the ability of mutants to survive a period of complete starvation. All studies were performed using *Canton-S* wild-type flies (*CanS*) and *DHR96*^{1} null mutants that had been crossed to *CanS* through nine generations of free recombination. Mature male flies were collected as 5- to 7-day-old adults, transferred to starvation media, and the number of living animals scored every 4 hours. *DHR96*^{1} mutants die at a faster rate than the wild-type controls (Figure 1A). We also tested *DHR96*^{2X} animals, which carry two transgenic copies of *DHR96* along with the endogenous wild-type locus. These flies express approximately 2-fold more DHR96 protein than wild-type animals (M. Horner, personal communication). The *DHR96*^{2X} animals are more resistant to starvation than the wild-type controls (Figure 1A). Taken together, these opposite effects of *DHR96* loss of function and gain of function on the starvation response suggest that this factor plays a central role in maintaining energy homeostasis.

As a first step toward determining the basis for these effects on the starvation response, we measured the major forms of stored energy in the animal, glycogen and TAG levels, in control, *DHR96*^{1}, and *DHR96*^{2X} stocks, under both fed conditions and after 20 hr of starvation. Both the *DHR96*^{1} mutant and *DHR96*^{2X} animals have wild-type levels of glycogen under normal feeding conditions (Figure 1B). The mutant, however, has significantly less glycogen upon starvation than the wild-type control. Interestingly, TAG levels are reduced in both fed and starved *DHR96*^{1} mutants and are elevated in both fed and starved *DHR96*^{2X} animals relative to controls (Figure 1C). An ~2-fold reduction in TAG is also seen with a second *DHR96* null allele and is effectively rescued by a wild-type genomic *DHR96* transgene (see Figure S1 available online). Taken together, these observations suggest that the starvation sensitivity of *DHR96* mutants is caused by a deficit of stored energy in the form of TAG, and implicate a central role for *DHR96* in TAG homeostasis.

To confirm and extend these results, we examined the distribution of neutral lipids in the major sites of fat storage in the animal, the fat body and midgut. Fat body cells were dissected from 1- to 2-day-old control male flies, *DHR96*^{1} mutants, and the *DHR96*^{2X} overexpression strain and stained with Nile red (Figures 1D–1F). *DHR96* mutant fat body cells are approximately half the size of those in controls, with an average ~3-fold reduction in lipid droplet size (Figures S2A and S2B). Conversely, fat body cells from the *DHR96*^{2X} overexpression strain are wild-type in size but have lipid droplets that are, on average, nine times larger than those in control fat cells (Figures S2A and S2B). Similar results were seen when the midguts of control, *DHR96*^{1} mutant, and *DHR96*^{2X} animals were dissected and stained with oil red O (Figures 1G–1I). Whereas low levels of neutral lipids can be detected in the gut epithelium of control flies (Figure 1G), no staining is evident in the gut epithelium of *DHR96*^{1} mutants (Figure 1H), and large lipid droplets are present in the midguts of *DHR96*^{2X} animals (Figure 1I). These results support our measurements of whole-animal TAG levels and indicate that *DHR96* plays a central role in the process of lipid metabolism.

DHR96 Mutants Are Resistant to Diet-Induced Obesity

We next tested whether dietary conditions can alter the TAG levels in *DHR96* mutant flies. Control *CanS* and *DHR96*^{1} mutants

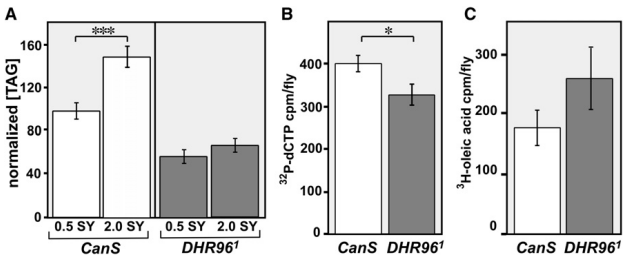

Figure 2. *DHR96* Mutants Are Resistant to Diet-Induced Obesity

(A) Mature adult male *Canton-S* (*CanS*) control flies and *DHR96*^{1} mutants were subjected to either a low-calorie 0.5 SY or high-calorie 2.0 SY diet for 7 days, after which TAG levels were determined. TAG levels were normalized for total protein and are presented as normalized to a wild-type level of 100%.

(B) Food uptake is slightly reduced in *DHR96* mutants, as determined by scintillation counting of *Canton-S* (*CanS*) control flies and *DHR96*^{1} mutants that were maintained on food supplemented with ^{32}P-dCTP.

(C) *DHR96* mutants can efficiently absorb dietary fatty acids, as determined by scintillation counting of *Canton-S* (*CanS*) control flies and *DHR96*^{1} mutants that were maintained on food supplemented with ^{3}H-oleic acid ($p = 0.18$).

Error bars represent ±SE, $^{*}p < 0.05$ and $^{***}p < 0.0001$.

were transferred to a previously characterized low-calorie (0.5 SY) or high-calorie (2.0 SY) medium and assayed for specific changes in TAG levels after 1 week (Mair et al., 2005). Control flies maintained on the high-calorie diet display a significant increase in whole-animal TAG levels relative to that seen in flies maintained on the low calorie diet (Figure 2A). Interestingly, *DHR96* mutants grown on either the low-calorie or high-calorie media display a lean phenotype relative to control animals on the restrictive diet. These observations support the proposal that *DHR96* mutants are genetically lean and indicate that they are resistant to diet-induced obesity.

It is possible that a reduced feeding rate could contribute to the lean phenotype in *DHR96* mutants. To test this possibility, both control and *DHR96*^{1} mutant flies were transferred to medium supplemented with a radioactive dCTP tracer, and the amount of retained label was measured after a 12 hr period (Carvalho et al., 2005). *DHR96* mutants display a mild (12%–18%) decrease in the amount of ingested food relative to the controls (Figure 2B). No change in food content, however, was seen upon spectrophotometric measurement of extracts from flies grown on yeast paste supplemented with 0.05% Bromophenol blue (data not shown). Based on these assays, we conclude that a reduction in feeding rate is unlikely to be a major cause of the lean phenotype in *DHR96* mutants. Similarly, there is no significant change in the amount of radiolabeled oleic acid retained in *DHR96* mutants after a 12 hr feeding period, suggesting that they have no major defects in dietary fatty acid uptake (Figure 2C).

DHR96 Mutants Are Resistant to Orlistat Treatment

We next wanted to determine if dietary TAG breakdown contributes to the lean phenotype in *DHR96* mutants. As a first step toward this goal, TAG levels were measured in control and *DHR96*^{1} mutants that were maintained for 5 days on media supplemented with 2 μM Orlistat (Figure 3A). Orlistat (tetrahydrolipstatin) is a widely used over-the-counter weight loss drug (Heck et al., 2000). It acts inside the intestine as a competitive inhibitor of pancreatic and gastric lipases, preventing their interaction with dietary TAG and thus blocking fatty acid release and dietary lipid uptake. Control flies exposed to Orlistat display a significant reduction in fat levels (Figure 3A). A similar effect was seen in mutants for the fly perilipin homolog Lsd-2, which are lean due to reduced levels of fat body TAG, consistent with the conclusion that Orlistat specifically inhibits *Drosophila* gastric lipases (Grönke et al., 2003) (Figure S3A). In contrast, no effect was seen upon exposing *DHR96* mutants to the drug. These observations were confirmed by staining midguts dissected from either untreated or Orlistat-treated control and *DHR96*^{1} mutants

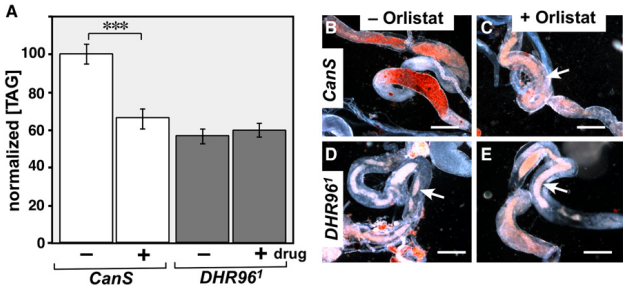

Figure 3. *DHR96* Mutants Are Resistant to Treatment with Orlistat

(A) Mature adult male *Canton-S* (*CanS*) control flies and *DHR96*^{1} mutants were transferred to a diet either with or without 2.0 μM Orlistat for 5–7 days, after which TAG levels were determined. TAG levels were normalized for total protein and are presented as normalized to a wild-type level of 100%. Error bars represent ±SE, $^{***}p < 0.0001$.

(B–E) Oil red O staining of dissected adult midguts shows that lipid accumulation is significantly reduced in *CanS* flies fed Orlistat (C), relative to controls (B) and undetectable in *DHR96*^{1} mutants maintained on either normal food (D) or food supplemented with Orlistat (E). Low levels of lipids can be detected in the midgut lumen of *DHR96* mutants and *CanS* flies fed Orlistat (arrows). Scale bars are 400 μm.

with oil red O (Figures 3B–3E). Control flies maintained on the high-nutrient diet used for this study display clearly detectable levels of neutral lipids in their gut epithelium, and this level is significantly reduced in the midguts of animals exposed to Orlistat (Figures 3B and 3C). In contrast, *DHR96* mutants display no detectable neutral lipids in their midguts, either in the presence or the absence of drug (Figures 3D and 3E). These results raise the possibility that the lean phenotype in *DHR96* mutants may arise, at least in part, from decreased TAG lipase activity in the midgut.

DHR96 Functions in the Midgut to Control the Breakdown of Dietary TAG

To test the possibility that *DHR96* mutants have a decreased ability to break down dietary fat, lysates were prepared from dissected control *CanS* and *DHR96*^{1} mutant midguts and assayed for TAG lipase activity by examining their ability to cleave a glycerol tributyrate substrate. This experiment showed that lysates from *DHR96* mutants have significantly reduced lipase activity relative to lysates from control animals, suggesting that decreased gastric lipase activity is a primary cause of the lean phenotype in *DHR96* mutants (Figure 4A). To further test this model, we attempted to bypass the need for midgut lipolysis by rescuing the lean phenotype through dietary supplementation with free fatty acids. Control *CanS* and *DHR96*^{1} mutant flies were transferred to a lipid-depleted medium that was either unsupplemented with lipid or supplemented with a mixture of stearic acid and oleic acid (free fatty acids) or a mixture of glycerol tristearate and glycerol trioleate (TAG). Extracts were prepared from these animals after 7 days and assayed for TAG levels (Figure 4B). As expected, *DHR96* mutants maintained on the lipid-depleted medium continue to display the lean phenotype. Interestingly, the same phenotype is seen when *DHR96* mutants are maintained in the presence of supplemented TAG, while the lean phenotype is effectively rescued by dietary supplementation with free fatty acids (Figure 4B). This observation indicates that an inability to break down dietary TAG is a major contributing factor to the lean phenotype seen in *DHR96* mutants. Phospholipid digestion, however, appears to be normal in *DHR96* mutants, as revealed by cleavage of the quenched fluorescent phospholipid PED6, which provides an accurate indicator of gastric phospholipase activity (data not shown) (Farber et al., 2001).

Expression of wild-type *DHR96* effectively rescues the lean phenotype of *DHR96* mutants, indicating that this phenotype can be attributed to a specific defect in *DHR96* function (Figure S1). If, however, *DHR96* regulates the breakdown of dietary fat, then we should be able to rescue the mutant lean phenotype by specifically expressing the wild-type receptor in the midgut of *DHR96* mutants. Consistent with this proposal, specific expression of wild-type *DHR96* in the fat body of *DHR96* mutants does not have a significant effect on the low levels of TAG seen in these animals, while specific expression of wild-type *DHR96* in the midgut of *DHR96* mutants effectively rescues the lean phenotype (Figure 4C). This observation indicates that the reduced levels of TAG seen in *DHR96* mutants arise from defects in midgut function. Taken together with our dietary rescue experiments, we conclude that *DHR96* plays a central role in controlling the breakdown of dietary fat.

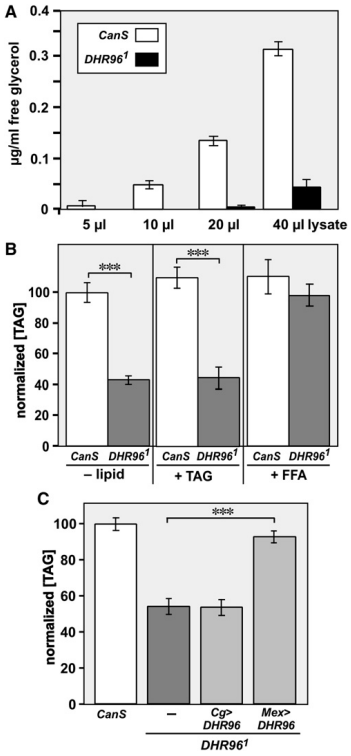

Figure 4. *DHR96* Functions in the Midgut to Control Dietary TAG Breakdown

(A) Midguts dissected from mature adult male *Canton-S* (*CanS*) control flies and *DHR96*^{1} mutants were homogenized, and increasing amounts of lysate were tested for TAG lipolytic activity by assaying for the release of glycerol from a glycerol tributyrate emulsion.

(B) Mature adult male *Canton-S* (*CanS*) control flies and *DHR96*^{1} mutants were transferred to lipid extracted 1.0 SY medium alone (−lipid), medium supplemented with TAG (+TAG), or medium supplemented with free fatty acids (+FFA) for 7 days, after which TAG levels were determined. TAG levels were normalized for total protein and are presented as normalized to a level of 100% in *CanS* flies on the unsupplemented lipid-extracted medium.

(C) Tissue-specific expression of a wild-type *UAS-DHR96* transgene in the midgut of *DHR96* mutants, driven by *Mex-GAL4* (*Mex* > *DHR96*), is sufficient to rescue the lean phenotype. In contrast, little effect is seen when *Cg-GAL4* is used to drive fat body-specific expression of the *UAS-DHR96* transgene (*Cg* > *DHR96*). The presence of the GAL4 drivers alone or the *UAS-DHR96* transgene in the *DHR96* mutant background has no effect on TAG levels (data not shown). Error bars represent ±SE, ***$p < 0.0001$.

DHR96 Regulates Genes Expressed in the Midgut and Involved in Metabolism

Microarray studies were conducted to determine the molecular mechanisms by which *DHR96* regulates lipid homeostasis.

This study revealed that 136 genes are significantly affected by the *DHR96* mutation in fed adult flies, with 94 genes displaying increased levels of expression and 42 genes showing decreased levels of expression (Table S1). A significant number of genes involved in cuticular structure and the peritrophic matrix, which acts as protective layer inside the lumen of the midgut, are upregulated in the mutant, while many genes that encode predicted α-mannosidases and endopeptidases are expressed at lower levels. Genes more directly involved in metabolism are also overrepresented in the list of *DHR96*-regulated genes. These include two genes that encode larval serum proteins: *Lsp1γ* (+2.2-fold in the *DHR96* mutant) and *Lsp2* (+3.1-fold). These proteins are synthesized by the fat body and are thought to provide a source of amino acids to support adult development during metamorphosis (Telfer and Kunkel, 1991). Interestingly, the gene that encodes the larval serum protein receptor, *Fbp1* (+3.1-fold), is also regulated by *DHR96*, suggesting a central role for *DHR96* in LSP function (Burmester et al., 1999). *Mdr50*, which is reduced in its expression in *DHR96* mutants (−1.5-fold), is the fly ortholog of mammalian ATP-binding cassette subfamily B, member 4 (ABCB4), a protein that is primarily expressed in the liver and involved in phospholipid transport. Similarly, the fifth most downregulated gene in *DHR96* mutants, *CG13325*, encodes a protein with a predicted acyltransferase domain that could function in lipid transport. Finally, it is remarkable that the most significantly affected genes in *DHR96* mutants include most of the *Drosophila* orthologs of the Niemann-Pick (NPC) disease genes that play central roles in cholesterol metabolism, including *npc1b* (+2.6-fold up) and five NPC2 family members: *npc2c* (+1.7-fold); *npc2d* (−13-fold); *npc2e* (+21-fold); and two genes that are below our 1.5-fold cutoff in expression level, *npc2g* and *npc2h* (both −1.4-fold).

Closer examination of the *DHR96*-regulated genes reveals that many are expressed exclusively, or most abundantly, in the midgut. Of the 132 genes that are misregulated in *DHR96* mutants, 42 are included in the 2695 midgut-specific genes identified by Li et al. (2008) (32%; p value = 9×10^{-9}). A similar overrepresentation of midgut-expressed genes can be seen by surveying the expression patterns on FlyAtlas (Chintapalli et al., 2007). For example, 12 of the 15 most downregulated genes, and 9 of the 15 most upregulated genes in *DHR96* mutants, are highly expressed in the midgut (Figure S4).

Finally, we found that many of the *DHR96*-regulated genes are located next to one another in the genome. The gene clusters include three genes with predicted α-mannosidase activity, *CG9463* (−3.3-fold), *CG9466* (−5.9-fold), and *CG9468* (−4.3-fold), all of which are among the most highly downregulated genes in the mutant. A divergently transcribed pair of genes with predicted α-mannosidase activity are also misregulated in *DHR96* mutants, *CG5322* (−1.3-fold) and *CG6206* (−1.8-fold), as well as two genes with predicted α-glucosidase activity, *CG14934* (+1.5-fold) and *CG14935* (−1.6-fold), and a cluster of two *Jonah* genes that encode predicted gastric peptidases, *Jon65Ai* and *Jon65Aii* (both −1.7-fold). Two genes with predicted sphingomyelin phosphodiesterase activity, *CG15533* (+2.0-fold) and *CG15534* (+1.3-fold), are regulated by *DHR96*. Moreover, many of the NPC genes that are misregulated in *DHR96* mutants lie within gene clusters. These include *npc2d* (−13-fold), *npc2e* (+21-fold), and *npc2c* (+1.7-fold). An adjacent gene, *fancl*, which has no known function, is downregulated 1.5-fold in *DHR96* mutants. In addition, *npc2g* and *npc2h* are located next to one another in the genome.

DHR96 Regulates the CG5932 Gastric Lipase to Promote TAG Accumulation

Two genes that encode predicted TAG lipases were identified in our microarray study of *DHR96* mutants, *CG5932* and *CG31091* (Table S1). Both genes encode members of the α/β-hydrolase fold lipase family and are homologs of human gastric lipases, with 37% and 33% amino acid identity, respectively. In addition, both genes are expressed almost exclusively in the larval and adult midgut (Chintapalli et al., 2007), raising the possibility that their misexpression may contribute to the reduced ability of *DHR96* mutants to break down dietary TAG. Validation of the effects of the *DHR96* mutation on *CG5932* and *CG31091* expression by northern blot hybridization showed that *CG31091* mRNA levels are very low, consistent with the *CG31091* expression levels reported on FlyAtlas (Chintapalli et al., 2007) (data not shown). In contrast, *CG5932* is abundantly expressed and is downregulated by starvation in wild-type flies (Figure 5A, lanes 1 and 3), and this expression is significantly reduced in fed or starved *DHR96* mutants (Figure 5A, lanes 2 and 4). *CG5932* is also the fourth most highly downregulated gene identified in our microarray study. Chromatin immunoprecipitation of DHR96 protein from wild-type lysates revealed direct binding to sequences immediately upstream from *CG5932* (Figure S5). This binding is not seen at a promoter that is independent of DHR96 regulation or in chromatin immunoprecipitation experiments using lysates made from *DHR96* mutants, suggesting that it represents a specific DNA-protein interaction. Moreover, expression of wild-type *DHR96* in the midgut of *DHR96* mutants is sufficient to restore normal *CG5932* expression (data not shown). Taken together, these observations suggest that *DHR96* maintains TAG homeostasis through direct regulation of *CG5932* expression.

If *CG5932* is essential for TAG homeostasis, then disruption of *CG5932* function should lead to changes in TAG levels. To test this possibility, we used *Act-GAL4* to drive the expression of a *UAS-CG5932* RNAi construct. Whereas flies that carry either the *Act-GAL4* driver alone or the *UAS-CG5932* RNAi construct alone display normal levels of TAG and *CG5932* expression, combining *Act-GAL4* with the *UAS-CG5932* RNAi construct resulted in significant reduction of both whole-animal TAG levels and *CG5932* mRNA accumulation (Figure 5B). Similar results were seen upon driving *UAS-CG5932* RNAi with the midgut-specific *Mex-GAL4* driver (Figure S6A). These animals are also sensitive to starvation, consistent with their lean phenotype (Figure S6B). In addition, purified CG5932 protein has lipolytic activity in vitro, as demonstrated by its ability to break down a glycerol tributyrate substrate (Figure S7). Taken together, these observations indicate that *CG5932* plays a critical role in the midgut to regulate whole-animal TAG homeostasis, most likely through its effect on the breakdown of dietary fat.

If the downregulation of *CG5932* expression in *DHR96* mutants contributes to their inability to break down dietary TAG, then restoring *CG5932* expression specifically in the midgut of these animals should rescue their lean phenotype. To test this possibility, we established transformant lines that

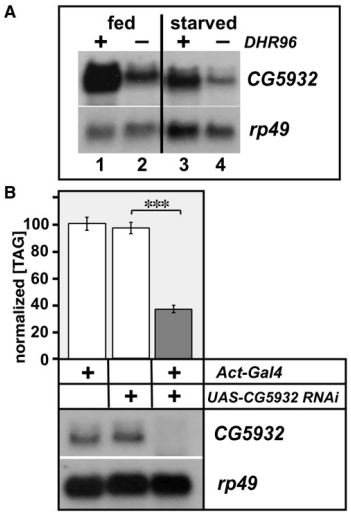

Figure 5. *DHR96* Regulates the *CG5932* Gastric Lipase Gene to Control the Breakdown of Dietary TAG

(A) RNA was isolated from either fed or starved *CanS* control flies (+ *DHR96*) and *DHR96*^{1} mutants (− *DHR96*) and analyzed by northern blot hybridization for *CG5932* transcription. Hybridization to detect *rp49* mRNA was used as a control for loading and transfer.

(B) An *Act-GAL4* driver or the *UAS-CG5932* RNAi transgene alone have no effect on whole-animal TAG levels (above) or *CG5932* mRNA levels (below), as determined by northern blot hybridization. Combining these two transgenes, however, effectively reduces *CG5932* expression and leads to a significant reduction in total TAG levels. Hybridization to detect *rp49* mRNA was used as a control for loading and transfer. Error bars represent ±SE, ***p < 0.0001.

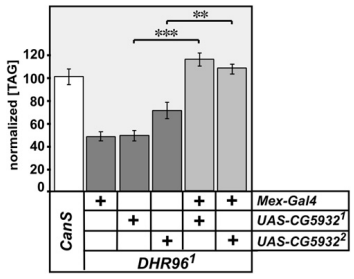

Figure 6. Midgut-Specific Expression of *CG5932* Rescues the Lean Phenotype of *DHR96* Mutants

TAG levels were determined in mature adult male *Canton-S* (*CanS*) control flies or *DHR96*^{1} mutants that carried different transgenes. Either the midgut-specific *Mex-GAL4* driver alone or two different insertions of the *UAS-CG5932* rescue transgene alone (*UAS-CG5932*^{1} or *UAS-CG5932*^{2}) had little or no effect on whole-animal TAG levels. The increased level of TAG seen in the presence of *UAS-CG5932*^{2} alone could be due to background expression from a flanking regulatory element, as is often seen with *P* element insertions. In contrast, combining the *Mex-GAL4* driver with each *UAS-CG5932* rescue transgene restored normal TAG levels. TAG levels were normalized for total protein and are presented as normalized to a wild-type level of 100%. Error bars represent ±SE, **p < 0.001 and ***p < 0.0001.

carry two different insertions of a *UAS-CG5932* transgene. As expected, the presence of either the midgut-specific *Mex-Gal4* driver alone or each *UAS-CG5932* transgene had little or no effect on the reduced TAG levels seen in the *DHR96* mutant (Figure 6). Combining the *Mex-GAL4* driver with either of the *UAS-CG5932* transgenes in a *DHR96* mutant, however, allows the mutant to recover normal levels of TAG (Figure 6). In addition, overexpression of *CG5932* does not stimulate an increase in whole-animal TAG levels, indicating that while *CG5932* is necessary for TAG homeostasis, it is not sufficient to drive TAG accumulation (Figure S8). These results, combined with our observation that the lean phenotype in *DHR96* mutants can be rescued by dietary supplementation with free fatty acids (Figure 4B), and the absence of an effect of Orlistat treatment in *DHR96* mutants (Figure 3A) or *Act > CG5932* RNAi animals (Figure S3B), suggests that *DHR96* controls whole-animal TAG levels through its regulation of the CG5932 gastric lipase.

DISCUSSION

Recent studies have implicated roles for mammalian PXR and CAR in controlling lipid metabolism, although little is known regarding the molecular mechanisms by which they exert these effects. Here we show that the single *Drosophila* ortholog of PXR and CAR, DHR96, plays an essential role in maintaining whole-animal TAG levels through the proper breakdown of dietary fat. Our results indicate that DHR96 acts through a previously uncharacterized gastric lipase encoded by *CG5932* to promote dietary lipid uptake and maintain TAG homeostasis.

DHR96 Mutants Are Sensitive to Starvation Due to Decreased Levels of TAG

Although *DHR96* null mutants are viable and fertile, with no morphological defects, they die significantly more rapidly than genetically matched control flies under starvation conditions, while *DHR96* overexpression leads to starvation resistance (Figure 1A). The effects of these genotypes on the major forms of stored energy in the animal, glycogen and TAG, are consistent with their effects on the starvation response. *DHR96* mutants have reduced levels of TAG under both fed and starved conditions, while *DHR96* overexpression leads to increased TAG levels (Figure 1C). Although no effects are seen on whole-animal glycogen levels in fed animals that either lack or overexpress *DHR96*, the mutants consume significantly more glycogen upon starvation than do controls (Figure 1B). This rapid utilization of glycogen stores is most likely due to the decreased energy contribution from TAG. Taken together, these observations suggest that the starvation sensitivity of *DHR96* mutants can be attributed to their lean phenotype, while the starvation resistance of the *DHR96*^{2X} strain is due to their excess energy in the form of TAG. This proposal is supported by the observation that genetically elevating the levels of TAG in *DHR96* mutants by introducing mutations in *bmm* or *AKHR*, which control distinct aspects of fat body TAG lipolysis (Grönke et al., 2007), effectively

rescues their starvation sensitivity (Figure S9). In addition, the opposite effects of *DHR96* loss of function and gain of function on both the starvation response and TAG levels argue that this receptor plays a central role in maintaining whole-animal TAG homeostasis.

DHR96 Functions in the Midgut to Regulate the Uptake of Dietary Nutrients

Several lines of evidence support the conclusion that *DHR96* exerts its primary metabolic functions through the midgut. These include our initial observation that *DHR96* mutants are resistant to treatment with the gastric lipase inhibitor Orlistat (Figure 3) and display reduced levels of midgut lipolytic activity (Figure 4A). In addition, dietary supplementation with free fatty acids, but not TAG, is sufficient to rescue the lean phenotype of *DHR96* mutants, as is midgut-specific expression of wild-type *DHR96* in a *DHR96* mutant background (Figure 4C). We also see a dramatic effect on lipid levels in the midgut, where almost no neutral lipids are detectable in *DHR96* mutants and enlarged lipid droplets are evident in $DHR96^{2X}$ flies maintained on a normal diet (Figures 1H and 1I). Interestingly, while the lumen of the midgut is not evident in control animals, we clearly see material in the lumen of *DHR96* mutant midguts or in control flies that are treated with Orlistat (Figures 1H and 3C–3E). In some cases, this material is stained by oil red O, suggesting that it may represent an increased level of undigested fat in these animals. This phenotype would be similar to that seen in humans who have defects in intestinal lipase activity (Ligumsky et al., 1990). Likewise, an increase in the passage of undigested dietary fat is a complication associated with Orlistat treatment in patients (Heck et al., 2000). Taken together, these observations support the conclusion that *DHR96* acts in the midgut to regulate the breakdown of dietary fat.

An essential role for *DHR96* in the midgut is further supported by our microarray study, which revealed that many *DHR96*-regulated genes are primarily expressed in this tissue. Interestingly, many of these genes have predicted roles related to the breakdown of dietary nutrients (Table S1). These include downregulation of multiple genes with predicted α-mannosidase activity, which is involved in the breakdown of the complex sugars found on glycoproteins. Many genes that encode trypsins and endopeptidases are also expressed at reduced levels in *DHR96* mutants as well as a few genes that encode predicted α-glucosidases, which are involved in the breakdown of dietary carbohydrates. In addition, a number of genes involved in the formation of the peritrophic matrix are more abundantly expressed in *DHR96* mutants. This matrix is comprised of chitin and peritrophic proteins and acts as a protective layer for the epithelial surface of the midgut (Hegedus et al., 2009). The peritrophic matrix also has critical roles in facilitating digestion. Only smaller molecules that arise from the initial digestion of complex nutrients, including peptides, sugars, and lipids, can move through the peritrophic matrix for final digestion and absorption by the midgut epithelium. These events are controlled by the selective partitioning of digestive enzymes to different sides of the peritrophic matrix as well as within the matrix itself. Thus, while midgut morphology appears normal in *DHR96* mutants, the effect of the mutation on the peritrophic matrix could impact nutrient digestion and absorption.

In addition to genes that regulate different aspects of lipid metabolism, our microarray study of *DHR96* mutants identified widespread effects on the expression of *Drosophila* homologs of NPC disease genes. The *npc1b* gene, which encodes an essential cholesterol transporter and the ortholog of mammalian NPC1L1 (Voght et al., 2007), is the tenth most highly upregulated gene in *DHR96* mutants. In addition, five of the eight *Drosophila* NPC2 genes are misregulated in *DHR96* mutants: *npc2c*, *npc2d*, *npc2e*, *npc2g*, and *npc2h* (Huang et al., 2007). These genes encode homologs of mammalian NPC2, which is involved in intracellular cholesterol trafficking (Huang et al., 2007). Remarkably, two of these genes are the most highly up- and downregulated genes identified in the mutant (*npc2e* and *npc2d*, respectively; Table S1). Moreover, many of these *npc* genes are located in clusters, suggesting that they are coregulated by the receptor. Although the function of these *npc2* genes is unknown, their disproportionate representation within the list of *DHR96*-regulated genes implies a critical role for the receptor in regulating cholesterol trafficking. Indeed, dietary cholesterol triggers a widespread transcriptional response in *Drosophila* that is dependent on *DHR96* function (Horner et al., 2009). Moreover, this study showed that *DHR96* mutants display defects in their ability to maintain cholesterol homeostasis when grown on a high-cholesterol diet. Taken together with the results presented here, our studies suggest that *DHR96* plays an essential role in the midgut to coordinate the processes of TAG and cholesterol breakdown, absorption, and trafficking.

DHR96 Regulates *CG5932* to Control the Breakdown of Dietary TAG

Two genes with predicted TAG lipase activity are expressed at lower levels in *DHR96* mutants (Table S1). One of these genes, *CG5932*, is abundantly expressed in the larval and adult midgut, encodes a protein that is highly related to human gastric lipase (LIPF, 37% identity over 358 amino acids), and is essential for maintaining whole-animal TAG levels (Figure 5). In addition, restoring *CG5932* expression in the midguts of *DHR96* mutants is sufficient to rescue their lean phenotype, defining this gene as a critical functional target of the receptor (Figure 6). Interestingly, *CG5932* expression is also regulated by starvation, with reduced expression in the absence of food and increased expression upon refeeding (Figure 5A) (Gershman et al., 2006). This regulation is consistent with an essential role for *CG5932* in the breakdown of dietary fat, where its expression is upregulated when food is present. This response, however, is unaffected in *DHR96* mutants, indicating that it is under independent control, possibly by known regulators of the starvation response such as dFOXO (Gershman et al., 2006).

The identification of *CG5932* as a key functional target of *DHR96* raises the question of how that regulation is achieved. We have tested a range of dietary parameters and candidate ligands using the GAL4-DHR96 ligand sensor but have been unable to identify conditions that activate the DHR96 LBD (Palanker et al., 2006). In addition, the DHR96-binding site remains undefined. DHR96 has a unique P box sequence within its DBD, which determines its DNA-binding specificity. This sequence is only shared by three *C. elegans* NRs, DAF-12, NHR-48, and NHR-8. Consistent with this observation, we found that DHR96 protein fails to bind to most canonical NR-binding

sites, except for weak binding to a palindromic EcR response element (Fisk and Thummel, 1995). One study has shown that DAF-12 displays preferential binding to a direct repeat of two distinct hexanucleotide sequences (AGGACA and AGTGCA), separated by five nucleotides (DR5) (Shostak et al., 2004). Whether DAF-12 contacts these sequences as a homodimer or a heterodimer with another NR, however, remains to be determined. The observation that many *DHR96*-regulated genes are arranged in clusters and DHR96 binds directly to a region upstream from the *CG5932* start site provides an ideal context for defining its DNA-binding specificity as well as determining the molecular mechanisms by which DHR96 coordinates target gene transcription.

DHR96 May Indirectly Regulate Xenobiotic Responses in *Drosophila*

Our studies of *DHR96* raise the interesting possibility that the defects in xenobiotic detoxification seen in *DHR96* mutants may arise, at least in part, from its role in regulating midgut metabolic activity. As noted in our original study, most of the genes that are regulated by phenobarbital in wild-type flies do so independently of *DHR96* (King-Jones et al., 2006). These genes include representatives of the major classes associated with xenobiotic detoxification: cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs), carboxylesterases, and UDP-glucuronosyl transferases (UGTs). Moreover, some phenobarbital-regulated genes that are misregulated in *DHR96* mutants still show a transcriptional response to the drug, although that response is muted. These observations indicate that one or more other factors contribute to the transcriptional response to xenobiotic challenge in *Drosophila*. Interestingly, similar results have been observed in vertebrates, where less then half of the genes that are regulated by xenobiotics are affected in *PXR* and *CAR* mutant mice (Maglich et al., 2002; Ueda et al., 2002). It remains unclear, however, whether this lack of regulation might be due to functional redundancy between these mammalian receptors.

There are several possible mechanisms by which the metabolic functions of *DHR96* could impact xenobiotic responses. First, a recent study of the expression patterns of *Drosophila* P450 genes showed that 34 of 60 genes that could be detected in third-instar larvae are expressed in the midgut or hindgut (Chung et al., 2009). A similar overrepresentation of P450 genes is evident in a microarray study of midgut expressed genes (Li et al., 2008). These observations indicate that, contrary to previous assumptions, the gut, and not the fat body, may be a critical site for xenobiotic detoxification (Chung et al., 2009). If this is true, then the sensitivity of *DHR96* mutants to phenobarbital or DDT treatment may be affected by the effects of this mutation on midgut physiology. This could occur through defects in the peritrophic matrix or through changes in the ability of the midgut to absorb lipophilic xenobiotic compounds such as DDT. An alternative possibility is that the sensitivity of *DHR96* mutants to xenobiotics might be due to their decreased energy stores. Detoxification requires energy expenditure. For example, P450s consume NADPH or NADH for their oxidation of xenobiotics, UGTs consume glucose, and GSTs consume glutathione. Thus, the reduced levels of stored energy in *DHR96* mutants might compromise their ability to properly inactivate toxic compounds. In addition, the reduced lipid stores in *DHR96* mutants might exert an indirect effect on xenobiotic responses by lowering the ability of the animal to sequester toxins in the fat reserves of the animal. Thus, there are multiple pathways by which the midgut-specific metabolic defects associated with the *DHR96* mutation might indirectly affect xenobiotic responses in these animals. Further studies are required to test this possibility and clarify the functional overlaps between the roles of *DHR96* in lipid metabolism and xenobiotic detoxification.

Conserved Roles for the PXR/CAR/DHR96 Nuclear Receptors in Lipid Metabolism

Several recent studies have demonstrated roles for both PXR and CAR in lipid metabolism (Moreau et al., 2008). CAR can repress the transcription of genes encoding carnitine palmitoyltransferase and enoyl-CoA isomerase, key steps in lipid β-oxidation (Ueda et al., 2002). *CAR* mutant mice are also sensitive to starvation, much as we observe for *DHR96* mutants, and lose weight more rapidly than wild-type mice when maintained on a low-calorie diet (Maglich et al., 2004). Transgenic expression of a constitutively active form of PXR in the mouse liver leads to hepatic steatosis along with reduced expression of lipid catabolic genes and increased expression of genes involved in lipid synthesis (Zhou et al., 2006). Importantly, similar effects were observed upon pharmacological activation of PXR using a specific agonist, indicating that the endogenous receptor can contribute to lipid homeostasis (Hoekstra et al., 2009; Nakamura et al., 2007). Most recently, a mutation in *CAR* has been shown to normalize the elevated serum TAG levels seen in *leptin*-deficient mice or in wild-type mice maintained on a high-fat diet (Maglich et al., 2009). Conversely, treatment of wild-type mice with a selective CAR agonist leads to increased serum TAG levels, and this response fails to occur in a *CAR* mutant background. Taken together, these studies indicate that activation of PXR/CAR receptors leads to lipid accumulation while a loss of PXR/CAR activity leads to reduced lipid levels, defining a central role for these NRs in lipid homeostasis. Their role in normal lipid metabolism, however, remains unknown, although it may be masked by functional redundancy between the two receptors. Similarly, the molecular mechanisms by which PXR and CAR can modulate lipid levels remain to be defined.

Our genetic studies of *DHR96* demonstrate that these metabolic activities of PXR and CAR have been conserved through evolution, and represent an essential ancestral function for this NR subfamily. Moreover, the observation that *DHR96* overexpression leads to lipid accumulation and *DHR96* mutants are lean suggests that the molecular mechanisms that underlie these effects are also conserved across species. This conclusion is supported by genetic studies of the *C. elegans* member of this subfamily, DAF-12, which indicate that this receptor is also required for proper levels of stored fat (Gerisch et al., 2001). Our study provides further evidence of a role for this NR subfamily in normal lipid homeostasis and defines the control of dietary fat breakdown as a key step at which this regulation is achieved. In addition, this work provides a foundation for understanding how dietary lipid uptake can impact normal lipid metabolism in *Drosophila* and provides a genetic model for characterizing how dietary factors can lead to lipid metabolic disorders such as obesity.

Cell Metabolism
Drosophila DHR96 Functions in Lipid Metabolism

EXPERIMENTAL PROCEDURES

Fly Stocks

The following stocks were used in this study: *DHR96*^{1} (King-Jones et al., 2006), *Cg-Gal4* (Hennig et al., 2006), *Mex-Gal4* (Phillips and Thomas, 2006), *Act-Gal4/CyO* (Bloomington # 25374), *bmm*^{1} (Grönke et al., 2005), *AKHR*^{2} (Grönke et al., 2007), *Lsd-2*^{KG00149} (Grönke et al., 2003), *w*^{1118}, *DHR96*^{2X} (M. Horner, personal communication), *UAS-DHR96* (M. Horner, personal communication), and *UAS-CG5932* RNAi (National Institute of Genetics stock 5932R-3). Flies were maintained on standard Bloomington Stock Center medium with malt at 25°C. The *UAS-CG5932 P* element construct was made using oligonucleotide primers 5′-ATAGAATTCATGAATCCAATCTTCTGCGC-3′ and 5′- ATACTC GAGCTAGCGACCTTCGTAGGAGT-3′ to amplify the *CG5932* cDNA (GenBank NM_140972) from the DGRC LP10120 cDNA clone by PCR. Following digestion with EcoRI and XhoI, the cDNA was inserted into the corresponding sites of the pUAST vector. This *UAS-CG5932* construct was then integrated into the *w*^{1118} genome.

Metabolic Assays

All studies used 1- to 2-day-old adult male flies that were aged 5–7 days prior to the experiment. Starvation sensitivity assays were conducted by transferring 15 samples of 20 flies of each genotype into vials containing 1% agar. Mortality was assayed every 4 hr as determined by a lack of responsiveness to touch. For glycogen and TAG assays, five to ten mature adult male flies were homogenized in 100–200 μl PBST (PBS, 0.1% Tween 20), heated at 70°C for 5 min to inactivate endogenous enzymes, and the homogenate cleared by centrifugation for 3 min. The supernatant was diluted 1:4 with PBST and assayed for glycogen levels as described (Palanker et al., 2009). TAG assays were conducted as described (Palanker et al., 2009), using the Stanbio triglyceride liquicolor kit (2100-430). The glycogen and TAG levels in each sample were normalized for total protein as determined by a Bradford assay. Both glycogen and TAG data were compiled from six samples collected from each genotype under each condition. All data are presented as normalized to a wild-type level of 100%. All assays were repeated three times, and a representative experiment is presented in each figure. Nile red staining was performed as described by Grönke et al. (2005), using Nile red mounting media (20% glycerol in PBS, with a 1:10,000 dilution of 10% Nile red in DMSO) and imaged on a Leica TCS SP2 confocal microscope using an excitation wavelength of 543 nm and a 600–650 nm emission spectrum. For oil red O stains, midguts from mature adult flies were dissected and fixed in 4% paraformaldehyde/PBS for 20 min. The midguts were washed with distilled water and incubated in 100% propylene glycol for 5 min. Specimens were then incubated at 60°C in oil red O stain (0.5% oil red O in propylene glycol), washed twice with propylene glycol, washed three times with PBS, and mounted in 20% glycerol/PBS for imaging.

Dietary Treatments

All studies used 1- to 2-day-old adult male flies that were aged 5–7 days prior to the experiment. Diet-induced obesity was assayed by transferring flies to either a low-calorie 0.5 SY diet or a high-calorie 2.0 SY diet for 7 days, after which extracts were prepared and assayed for TAG and protein (Mair et al., 2005). Food intake was measured by transferring flies to Bloomington cornmeal, yeast, and molasses medium supplemented with 0.1 μC/ml ^{32}P-dCTP (Perkin-Elmer) for 12 hr, after which they were transferred to normal unlabeled food for 4 hr to remove label that was nonspecifically bound to the outside of the animal (Carvalho et al., 2005). Ten to twelve groups of 20 flies were collected, washed once with 0.1% BSA, and assayed for label retention on a scintillation counter. Fatty acid uptake was determined in a similar manner, using ^{3}H-oleic acid (Moravek Biochemicals) at a final concentration of 3 μC/ml. Treatment with Orlistat was conducted by growing flies on a high-nutrient molasses medium (Bloomington cornmeal, yeast, and molasses media) with or without 2 μM Orlistat for 5–7 days. TAG levels were then assayed as above. All data shown are from two parallel data sets of six samples/stock/condition compiled together and repeated at least three times. Dietary lipid supplementation was performed by transferring mature adult males to lipid-depleted 1.0 SY for 5–7 days. This medium was prepared by extracting the yeast extract and agar components of the SY medium overnight with chloroform, followed by a second 4 hr chloroform extraction. Both components were then allowed to dry for 2–3 days in a fume hood. The lipid-depleted medium was supplemented with either free fatty acids (5 mg/ml stearic acid and 5 mg/ml oleic acid, ChemService) or TAG (5 mg/ml glycerol tristearate and 5 mg/ml glycerol trioleate, ChemService). All data shown are from two collections of six samples collected from each genotype under each condition. Each experiment was repeated at least three times.

Microarrays

Adult male *CanS* and *DHR96*^{1} flies were collected 1–2 days after eclosion and allowed to age for 7 days on normal medium. RNA was extracted from these animals using Trizol (GIBCO) and purified on RNAeasy columns (QIAGEN). All samples were prepared in triplicate to facilitate subsequent statistical analysis. Probe labeling, hybridization to Affymetrix GeneChip *Drosophila* Genome 2.0 Arrays, and scanning were performed by the University of Maryland Microarray Core Facility. Raw data were subjected to quantile normalization using R statistical analysis software, and gene expression changes were determined using SAM 2.0, with a <5% estimated false positive rate and a 1.5-fold cutoff in expression level (Tusher et al., 2001). Comparison between microarray data sets was performed using Microsoft Access.

Statistical Analyses

Statistical significance was calculated using an unpaired two-tailed Student's t test with unequal variance. All quantitative data are reported as the mean ± SEM.

ACCESSION NUMBERS

Microarray data from this study can be accessed at the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) under accession number GSE18576.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, one table, and nine figures and can be found with this article online at http://www.cell.com/cell-metabolism/supplemental/S1550-4131(09)00336-2.

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CHAPTER 3

COORDINATION OF TRIGLYCERIDE AND CHOLESTEROL HOMEOSTASIS BY *DHR96* AND THE *DROSOPHILA* *LIPA* HOMOLOG *MAGRO*

Introduction

Coordinate regulation of lipid metabolism is central to human health, with disruption of this process leading to a range of metabolic disorders, including obesity and cardiovascular disease. Normal lipid homeostasis is maintained by balancing dietary lipid uptake and synthesis with lipid catabolism and excretion. Under normal feeding conditions, dietary lipids such as triacylglycerol (TAG) and cholesterol esters are broken down into free fatty acids, monoacylglycerols, and free sterols in the lumen of the intestine. These digested lipids can then be absorbed by the intestinal cells, where TAG is resynthesized and packaged together with cholesterol, cholesterol esters, and carrier proteins to form lipoprotein particles that are released into the circulatory system to be trafficked throughout the body. These trafficked lipids can be either utilized by cells or deposited in storage tissues such as the adipose and liver. Under conditions of excess lipids, stored TAG and cholesterol esters are broken down and free fatty acids can be utilized for energy, while excess cholesterol is modified into polar sterols in the liver and excreted from the intestine (Ikonen, 2008; Lusis and Pajukanta, 2008).

Nuclear receptors (NRs) are ligand-regulated transcription factors that play essential roles in multiple aspects of lipid homeostasis. Many NRs bind small lipophilic compounds such as fatty acids, sterols, and other metabolic intermediates, and coordinate multiple aspects of metabolism by directing specific changes in gene expression. One example of this is the LXR/FXR subclass of NRs that play central roles in TAG metabolism and cholesterol homeostasis. Previous studies of LXRa (NR1H3) in mice have shown this receptor binds oxysterols and promotes the modification and clearance of excess sterols by cholesterol efflux and reverse cholesterol transport (Kalaany and

Mangelsdorf, 2006). In addition, LXRa is required to maintain proper TAG levels, at least in part through the regulation of SREBP-mediated fat synthesis (Schultz et al., 2000). LXRa activity is thus central to both TAG and cholesterol homeostasis, although much remains to be learned about the roles of specific LXR target genes in mediating these key metabolic functions.

We have been studying a *Drosophila* homolog of LXRa, DHR96, as a simple system to understand the physiological and molecular roles for this family of NRs and their target genes. Biochemical and genetic studies of DHR96 have shown that it shares the central metabolic functions of its mammalian counterpart. DHR96 binds cholesterol and is required for normal cholesterol homeostasis, with *DHR96* null mutants exhibiting a 20-30% increase in whole animal cholesterol levels due, at least in part, to increased *npc1b* expression (Horner et al., 2009; Bujold et al., 2010). In addition, *DHR96* mutants display an approximate 50% decrease in whole animal TAG levels. This decrease in TAG arises, in part, from an inability of the *DHR96* mutant to break down dietary TAG due to a major decrease in the expression of the intestinal lipase Magro *(CG5932)* (Sieber and Thummel, 2009). Interestingly, *magro* transcription is responsive to dietary cholesterol levels and this regulation is dependent on *DHR96* function, providing a potential link between cholesterol levels, DHR96, and TAG homeostasis (Horner et al., 2009; Bujold et al., 2010). Moreover, while Magro protein is most similar to mammalian gastric lipase (38% identity, 56% similarity), the second most homologous mammalian protein is LipA (32% identity 50% similarity), which has both TAG lipase and cholesterol esterase activities (Ameis et al., 1994). Consistent with these enzymatic functions, mutations in *LipA* lead to a major decrease in white adipose tissue along with elevated levels of stored

and circulating cholesterol in mice (Du et al., 1998; Du et al., 2001). *LipA* mutants also display excess cholesterol esters and TAG in the intestine and liver – hallmarks of Wolman's disease and Cholesterol Ester Storage Disease (CESD), which are caused by mutations in human *LipA* (Crocker et al., 1965; Kyriakides et al., 1970; Burke and Schubert, 1972). Taken together, these observations raise the possibility that, in addition to maintaining TAG homeostasis, *magro* may have a critical role in regulating cholesterol homeostasis, and that *DHR96* may function through *magro* to help coordinate TAG and cholesterol metabolism.

In this study we show that loss of *magro* function leads to a ~20-25% increase in whole animal cholesterol levels, similar to that seen in the *DHR96* mutant. Biochemical and genetic studies reveal that, like LipA, Magro has cholesterol esterase activity, and this enzyme is required in intestinal cells to clear excess cholesterol. In contrast, the TAG lipase activity of Magro arises from the anterior end of the gut and acts in the intestinal lumen to facilitate dietary fat uptake. Restoring *magro* expression in the intestine of the *DHR96* mutant is sufficient to fully rescue the lean phenotype and partially rescue the cholesterol accumulation defect seen in *DHR96* mutants. Our data support the model that *DHR96* functions through *magro* in the intestine to coordinate both dietary TAG breakdown and the clearance of excess sterols. These studies provide new insights into the mechanisms by which nuclear receptors can promote the clearance of excess sterols by transintestinal cholesterol efflux.

Materials and methods

Fly stocks

The following stocks were used in this study: *Canton S, DHR96*[1] (King-Jones et al., 2006), *Mex-Gal4* (Phillips and Thomas, 2006), *Act-Gal4/CyO* (Bloomington # 25374), *UAS-magro* (Sieber and Thummel, 2009), *UAS-DHR96* (Horner et al., 2009), *UAS-MAGRO-RNAi (NIG Fly), bab1-GAL4/TM3* (Cabrera et al., 2002). Flies were maintained on Standard Bloomington Stock Center medium with malt at 25°C.

magro-EGFP genomic transgenic line

The genomic *magro-EGFP* transgenic stock was made using oligonucleotide primers 5'- GAATTCGGCGCGCCCCACTAAACCACGCCGCTGTTG -3' and 5'-GAATTCCCGCGGTAAGCGACCTTCGTAGGAGT-3' to amplify a 6.32 kb genomic fragment spanning the *magro* locus, containing 5 kb of promoter sequence and the entire 1.3 kb protein coding region. The 3' end of this fragment contains a mutation that converts the TAG stop codon into a TTA codon to allow translational read though into EGFP. This fragment was digested with Asc1 and SacII and the resulting fragment was ligated into the corresponding sites of the pStinger P-element vector (Barolo et al., 2004). Oligonucleotide primers, 5'-GAATTCCCGCGGAAATCGGCCAATAAAAGAATCCG-3' and 5'- GAATTCACTAG**T**AGCCTTACGATATCTAATCGACTAAAG-3', were then used to amplify a 302 bp fragment that spans the endogenous 3'-UTR of *magro*. This fragment was digested with SpeI and SacII and cloned into the corresponding sites in the *pStinger-magro* vector. The EGFP coding sequence was then amplified from pStinger by PCR using oligonucleotide primers 5'-

GAATTCCCGCGGATGGTGAGCAAGGGCGAGG-3' and 5'-GAATTCCCGCGGCTACAGCTCGTCCATGCCGAGAG-3'. The resulting fragment was digested and ligated into the SacII site in the vector between the *magro* coding sequence and the 3' UTR, resulting in the genomic *magro-EGFP* translational fusion construct. This vector was integrated into the w^{1118} genome following standard methods.

Metabolite assays

Newly eclosed adult male flies were aged 5-7 days prior to use for all experiments. Pancreatin dietary supplementation was conducted by raising flies for their entire life cycle in the presence of 5 mg/ml pancreatin (Sigma), after which 7-day-old adult male flies were collected and assayed for TAG and cholesterol. TAG and cholesterol assays were conducted as described (Horner et al., 2009; Sieber and Thummel, 2009). All results shown are compiled from two parallel data sets from six samples collected from each genotype under each condition, and repeated at least three times. To measure cholesterol ester levels, 30 adult male flies were collected, washed twice with 1X PBS, and homogenized in 250 µl of 1X PBST. The total volume was brought to 1.0 ml with 1X PBST and the samples were divided into two 500 µl aliquots. Both aliquots were sonicated three times for 30 seconds, after which 10 µl of cholesterol esterase (Sigma) was added to one aliquot while the other aliquot served as an undigested control. Samples were incubated overnight at 37°C with periodic vortexing. Lipids were extracted using a 2:1 chloroform-methanol mixture and dried by vacuum centrifugation. Lipids were solubilized using 500 µl 1X PBST by vortexing, followed by sonication (6x30 seconds). A 25 µl lipid sample was added to 25 µl of Amplex reaction buffer in each well of a

black 96 well plate, followed by 90 µl of Amplex reagent (Amplex Red Cholesterol Assay Kit, Invitrogen). The plates were mixed and incubated at 37°C for 30 minutes. Samples were then assayed according to the kit instructions and measured in a Molecular Devices SpectraMax M2 fluorometer. The readings from undigested samples were subtracted from those of the digested samples to determine the relative levels of cholesterol esters. The data presented were compiled from three replicate samples per genotype and were repeated at least three times.

In vivo cholesterol esterase activity assay

Cholesterol ester substrate for the assay was made by adding 40 mg cholesterol acetate (Sigma) to 4 mls of Amplex reaction buffer (Invitrogen). The mixture was sonicated (6x30 seconds) to make a lipid suspension, incubated for 15 minutes at 50^0C, and centrifuged at 12,000 x G for 3 minutes to clear the suspension of insoluble cholesterol acetate. Intestines from 30 adult male flies were dissected and homogenized in 100 µl Amplex reaction buffer (Invitrogen) and centrifuged at 12,000 x G for 2 minutes to clear the lysate. Forty microliters of reaction buffer and 10 µl of cholesterol ester solution were added to each well in a black 96 well plate. Cleared lysate was then added to each well, except for the nondigested controls, and mixed by pipetting. Ninety microliters of Amplex reagent (Amplex Red Cholesterol Assay Kit, Invitrogen) was added to each well followed by a 30-minute incubation at 37^0C. Fluorescence for each well was then measured according to the kit instructions. The readings from undigested samples were subtracted from those of the digested samples to determine the amount of

free cholesterol released. These data presented were compiled from three replicate samples per genotype and were repeated at least three times.

Statistical analyses

Statistical analysis was done using an unpaired two-tailed Student's t-test with equal variance. All quantitative data are reported as the mean ± SEM.

Results

magro is required for normal cholesterol homeostasis

The regulation of *magro* transcription by dietary cholesterol combined with its significant homology to *LipA* prompted us to test if *magro* function is required for cholesterol homeostasis. *DHR96*^{1} null mutants grown on a normal diet display ~20-30% elevated levels of whole animal cholesterol compared to genetically matched wild-type controls (Figure 3.1A), similar to the results seen when *DHR96* mutant larvae are subjected to a high cholesterol diet (Horner et al., 2009). Interestingly, RNAi-mediated silencing of *magro* expression, as done previously (Sieber and Thummel, 2009), leads to a similar phenotype relative to the *Act-GAL4/+* and *UAS-magro RNAi/+* controls (Figure 3.1A). Taken together with our earlier work, which showed that both *DHR96* mutants and *magro* RNAi animals have significantly lower levels of TAG, these data suggest that *DHR96* functions through transcriptional regulation of *magro* to coordinate TAG and cholesterol homeostasis in *Drosophila*.

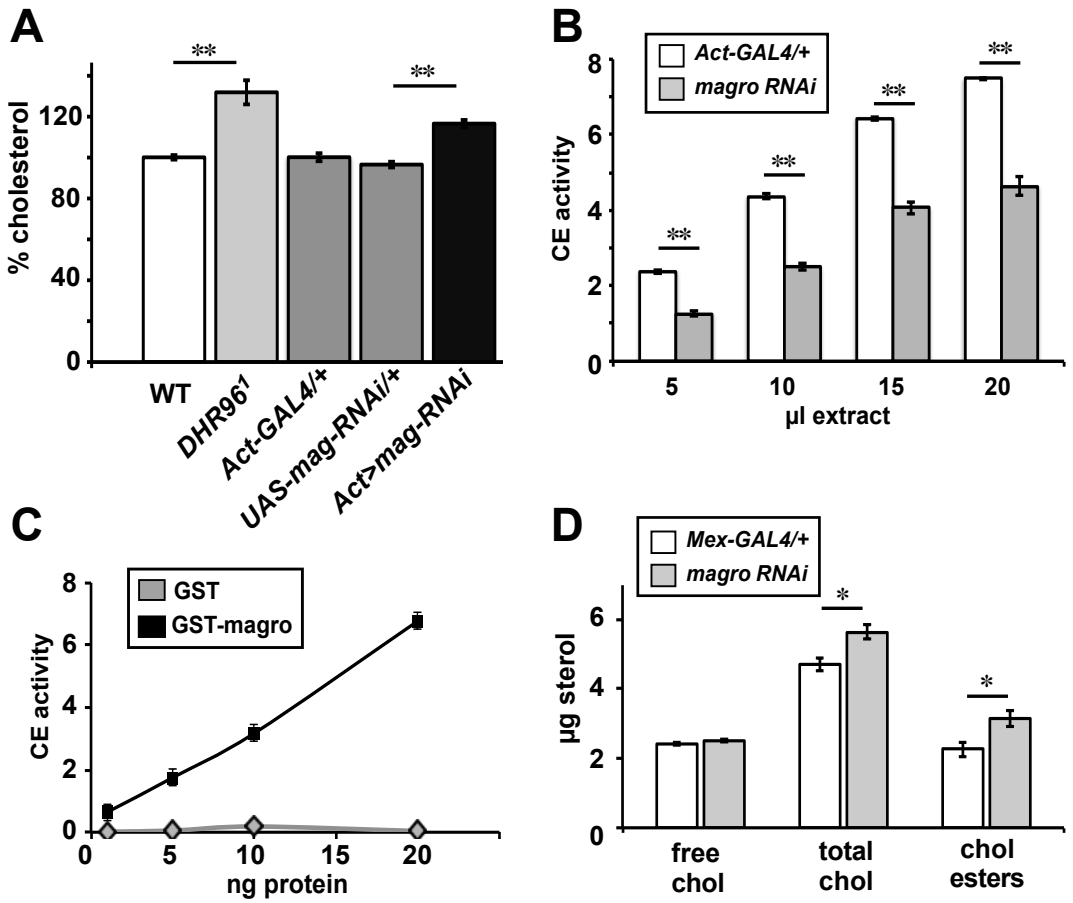


Figure 3.1 *magro* maintains proper cholesterol levels and has cholesterol esterase activity.

(A) RNAi for *magro* results in elevated cholesterol levels similar to those seen in *DHR96* mutants. Wild-type (WT), *Act-GAL4*/+, and *UAS-magro-RNAi*/+ control flies, along with *DHR96*^{1} mutants and *Act-GAL4*/*UAS-magro-RNAi* animals (*Act>RNAi*) were assayed for total cholesterol levels. These data were normalized to protein levels and are presented relative to a wild-type level of 100%. (B) RNAi for *magro* results in reduced intestinal cholesterol esterase activity. Intestines dissected from both *Act-Gal4/+* control and *Act-GAL4*/*UAS-magro-RNAi* (*magro* RNAi) animals were homogenized and increasing amounts of lysate were tested for cholesterol esterase (CE) activity by assaying for the release of free cholesterol from a cholesterol acetate substrate. (C) Purified Magro-GST protein has cholesterol esterase activity. Recombinant GST and Magro-GST were purified as described (Sieber and Thummel, 2009) and increasing amounts of protein were assayed for cholesterol esterase (CE) activity. (D) RNAi for *magro* results in elevated levels of esterified cholesterol. Free, esterified, and total cholesterol levels were measured from *Mex-GAL4/+* control and *Mex-GAL4/UAS-magro-RNAi* (*magro* RNAi) animals. Error bars represent ±SEM. (*$p < 0.05$, **$p < 0.0001$)

magro regulates cholesterol homeostasis by breaking down stored cholesterol esters

Because mammalian LipA is known to cleave cholesterol esters in addition to its TAG lipase activity, we tested if Magro shares these dual enzymatic functions (Ameis et al., 1994). The expression of *magro* was effectively silenced by RNAi and extracts from dissected intestines were tested for cholesterol esterase activity. While control intestinal lysates exhibit a high level of cholesterol esterase activity in a dose-dependent manner, the lysates from *magro* RNAi animals display a significant ~50% decrease in enzymatic function (Figure 3.1B). Moreover, purified recombinant Magro-GST efficiently cleaves a cholesterol ester substrate *in vitro*, demonstrating that these effects are a direct result of Magro enzymatic activity (Figure 3.1C). Thus, taken together with our earlier biochemical studies, this result shows that Magro is a bifunctional enzyme that can act as both a TAG lipase and cholesterol esterase (Sieber and Thummel, 2009).

If decreased cholesterol esterase activity in the intestine is the cause of the elevated cholesterol levels seen in the *magro* RNAi animals then we should see a specific increase in stored cholesterol esters in these animals. To test this possibility, we used the *Mex-GAL4* driver to specifically silence *magro* expression in the intestine and then measured free cholesterol, total cholesterol, and cholesterol esters (Figure 3.1D). Free cholesterol levels are the same in control and *Mex>magro* RNAi animals, and total cholesterol levels were elevated upon *magro* RNAi, similar to the result seen when the ubiquitous *Act–GAL4* driver is used to direct *magro* RNAi (Figure 3.1A). Importantly, cholesterol ester levels are also significantly higher in *Mex>magro* RNAi animals relative to the *Mex-GAL4/+* controls (Figure 3.1D). Taken together, these data support the model

that Magro maintains cholesterol homeostasis through its ability to directly break down stored cholesterol esters in the intestine.

magro is expressed throughout the digestive tract

Although microarray studies have demonstrated that *magro* is expressed specifically in the midgut, its expression within this tissue remains undefined (Drysdale, 2008). We therefore examined Magro expression in the digestive tract using a *magro-EGFP* transgenic line. This construct contains ~7 kb of genomic DNA spanning the *magro* locus with 5 kb of upstream promoter sequences and the EGFP gene fused in-frame to the 3'-end of the *magro* protein coding region. EGFP expression in these animals revealed that Magro is present throughout the intestine with the highest levels of protein accumulation in the anterior region of the proventriculus of the digestive tract (also called the cardia) (Figure 3.2A). The proventriculus is a bulb shaped structure that consists of three distinct cell layers (Figure 3.3). The esophagus, which enters from the anterior end and runs to the base of the proventriculus, folds back on itself to form a second, internal cell layer that runs back to the anterior end of the structure and again folds back on itself to form the outermost cell layer, which is continuous with the body of the intestine. Magro-EGFP is expressed in the anterior half of the outermost layer of cells in the proventriculus (Figure 3.2C, arrow). In addition, protein is clearly visible in large vesicles that lie posterior to this region of expression (Figure 3.2C-E, yellow boxes). These are acidic vesicles that stain positive for Lysotracker Red (Figure 3.2F-H) consistent with the acid lipase activity of Magro and LipA. Interestingly, visualization of these vesicles using CD8-EGFP reveals that they move in a posterior direction toward the

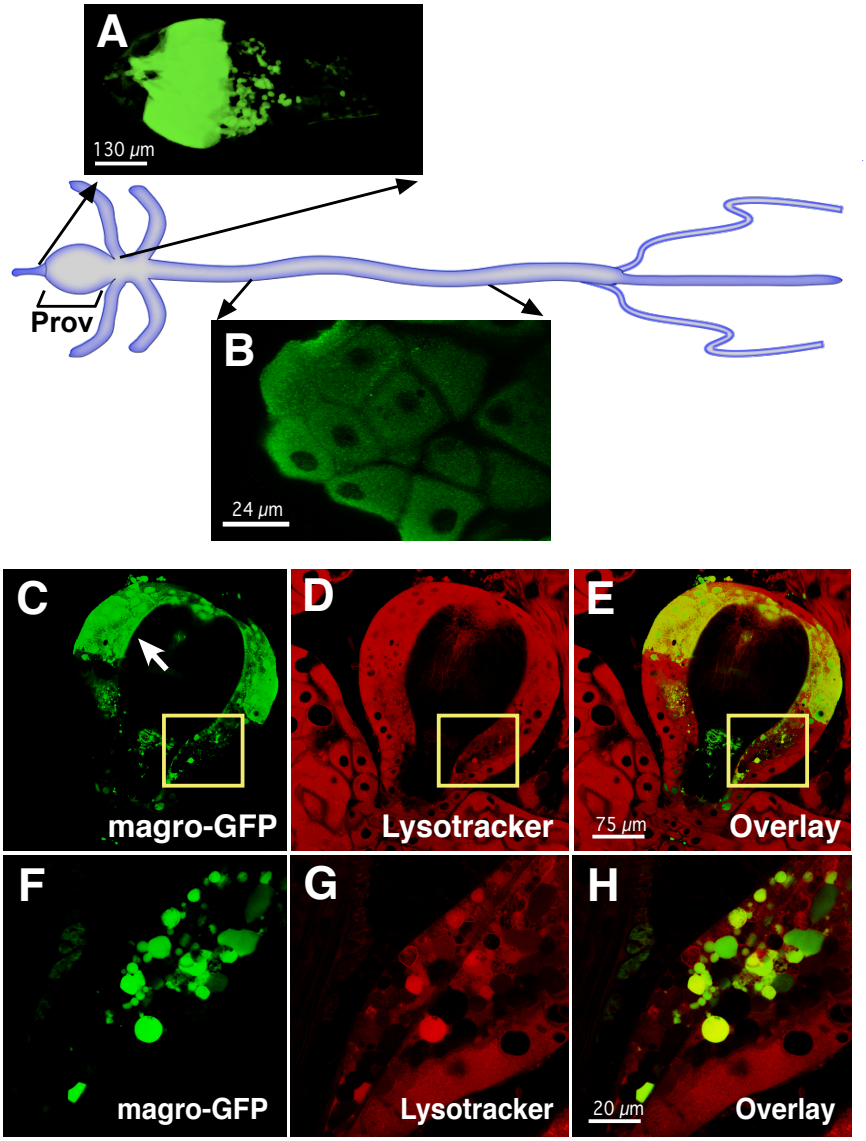


Figure 3.2 Magro is expressed in the proventriculus and midgut
A genomic *magro-EGFP* fusion construct was used to visualize the distribution of Magro expression in mid-third instar larvae. Magro-EGFP (green) expression is restricted to the intestine and accumulates to high levels in the anterior region of the proventriculus (A, arrow in C). Antibody staining for EGFP also revealed lower levels of punctate expression in the cytoplasm of enterocytes (B). Higher resolution images of the proventriculus revealed Magro-EGFP in large vesicles that extend from the abundant expression at the anterior end of the proventriculus toward the junction with the midgut lumen (yellow boxes in C,D,E, shown in panels F,G,H). Lysotracker Red stains the large acidic vesicles that contain Magro-EGFP protein (D,E,G,H).

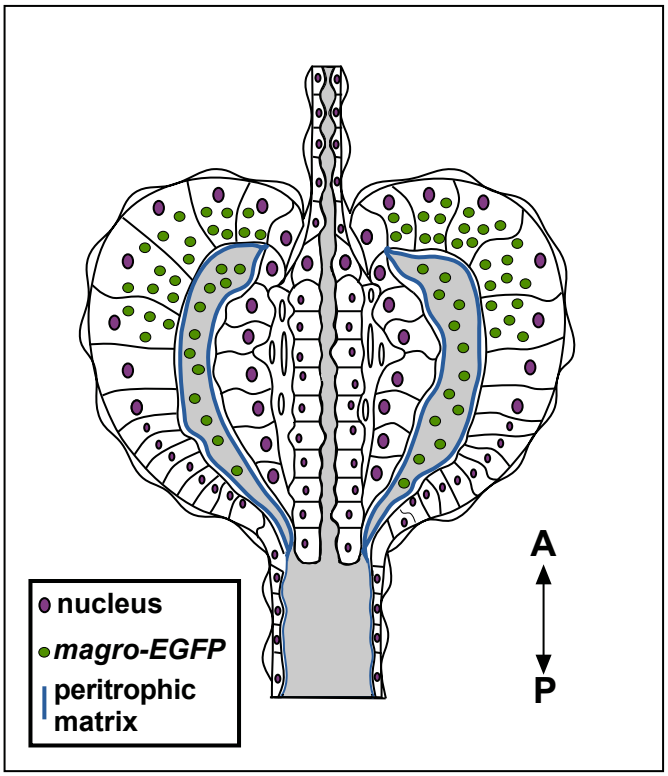


Figure 3.3 Schematic representation of the Drosophila proventriculus.
The proventriculus is a bulb shaped structure that consists of three distinct cell layers. The esophagus, which enters from the anterior end (top of figure) and runs to the base of the proventriculus, folds back on itself to form a second, internal cell layer that runs back to the anterior end of the proventriculus and again folds back on itself to form the outermost cell layer, which is continuous with the body of the intestine (King, 1988). magro-EGFP protein (green) is abundantly expressed in the anterior outermost cell layer, and released as vesicles that appear to be localized to the intercellular space where the peritrophic matrix is deposited.

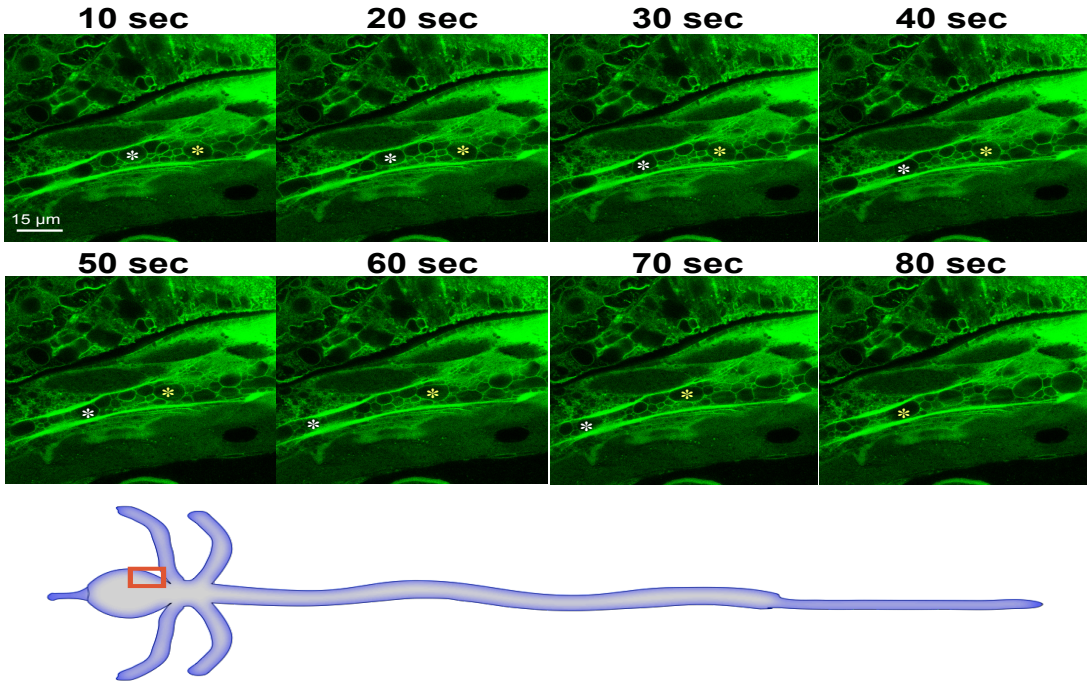


Figure 3.4 *CD8-GFP* positive vesicles move within the proventriculus *Tub-GAL4* was used to drive expression of the *UAS-CD8-GFP* membrane marker to visualize membrane-bound vesicles in the posterior region of the proventriculus (red box in diagram at bottom). Images were captured at 10 second intervals for 80 seconds, revealing rapid vesicle movement toward the base of the proventriculus. Two vesicles are marked with white and yellow asterisks.

intestine, providing a potential mechanism to deliver digestive enzymes, such as Magro, into the intestinal lumen (Figure 3.4). Lower levels of Magro-EGFP protein can also be seen in the major cell type of the intestine, the enterocytes, in a punctate cytoplasmic pattern (Figure 3.2B). Interestingly, we do not observe Magro-EGFP in large acidic Lysotracker-positive vesicles in these cells. Taken together, these observations suggest that Magro is trafficked differently in different cell types of the digestive tract and raise the possibility that that the cholesterol esterase and TAG lipase activities of this enzyme are spatially seperated.

magro acts within the intestinal lumen to maintain TAG homeostasis

As a first step toward determining if the enzymatic activities of Magro are regionally localized within the intestine, we examined if restoring Magro enzymatic functions in the lumen of the gut could rescue the lipid metabolic defects seen in the *DHR96* mutant and *magro* RNAi animals. Both stocks were grown on a diet supplemented with pancreatin, which is a mixture of pancreatic enzymes that includes TAG lipase and cholesterol esterase activities, after which whole animal TAG and cholesterol levels were determined. While pancreatin supplementation had no effect on TAG levels in either *Canton S* or *Act-GAL4/+* control animals, this diet restored wild-type levels of TAG in both *DHR96* and *magro* RNAi animals (Figure 3.5 A,B). Conversely, pancreatin supplementation had no impact on the elevated cholesterol levels seen in the *DHR96* or *magro* RNAi animals (Figure 3.5 C,D). These results are consistent with those seen when wild-type flies are treated with Orlistat (tetrahydrolipstatin), which acts as a competitive inhibitor of secreted lipases and cholesterol esterases inside the lumen of the intestine (Borgstrom, 1988). Although Orlistat treatment is sufficient to decrease whole animal TAG levels, as seen previously (Sieber and Thummel, 2009), no significant effect is seen on the levels of total cholesterol (Figure 3.6). These data confirm our earlier studies indicating that Magro functions in the intestinal lumen to maintain appropriate levels of TAG, and demonstrate that its effects on cholesterol homeostasis are conferred within the cells of the intestinal tract.

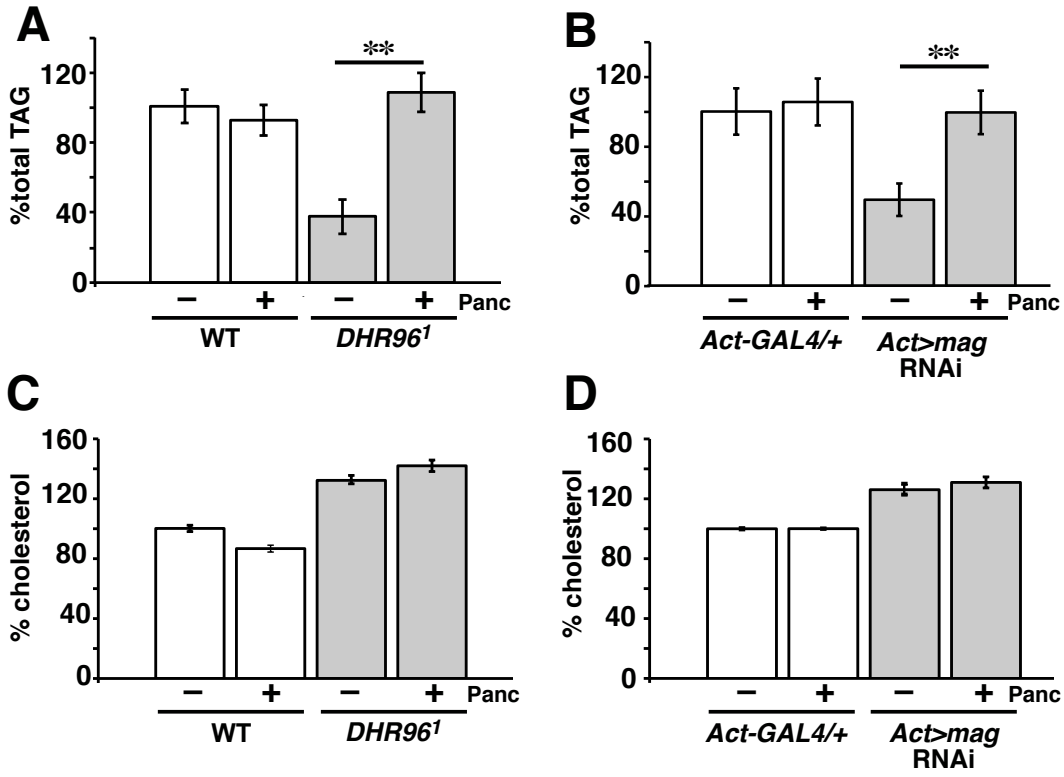


Figure 3.5 Magro enzymatic activity is not required in the intestinal lumen to maintain cholesterol homeostasis

Wild-type (WT) and *Act-GAL4*/+ controls, along with *DHR96*^{1} mutants and *Act-GAL4*/*UAS-magro-RNAi* (*mag* RNAi) animals, were raised on medium supplemented with 2 mg/mls pancreatin. Mature adults were collected and assayed for TAG (A,B) and cholesterol (C,D) levels. The data were normalized to protein levels and are presented relative to a wild-type level of 100%. Pancreatin supplementation is sufficient rescue the reduced TAG levels in both *DHR96* mutants and *magro* RNAi animals (A, B), but has no significant effect on their increased cholesterol levels (C,D). Error bars represent ±SEM. (**$p < 0.0001$)

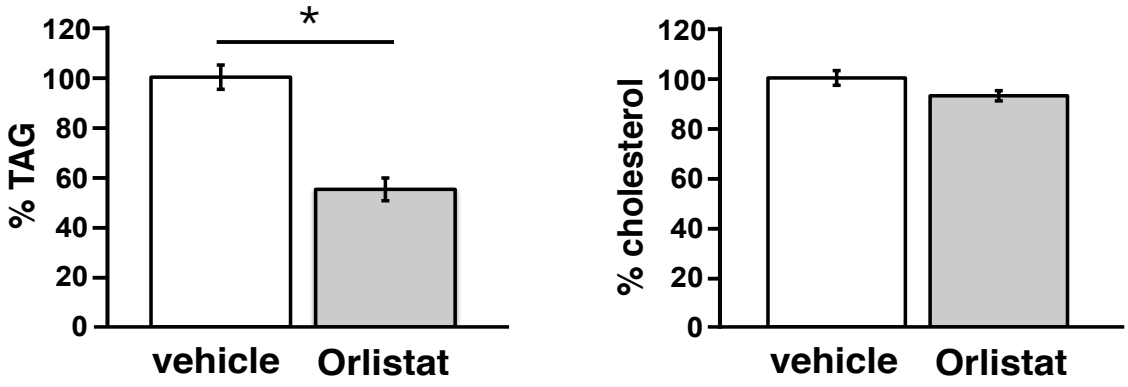


Figure 3.6 Orlistat treatment decreases TAG levels, but has no effect on cholesterol levels in wild-type flies

Mature adult male *Canton S* flies were transferred to a diet supplemented with 2.0 μM Orlistat for seven days, after which TAG and cholesterol levels were determined. The data was normalized to protein levels and are presented relative to a level of 100% for treatment with vehicle alone. These results were compiled from six replicate samples and repeated at least three times. Error bars represent +/- SEM. * $p < 0.0001$

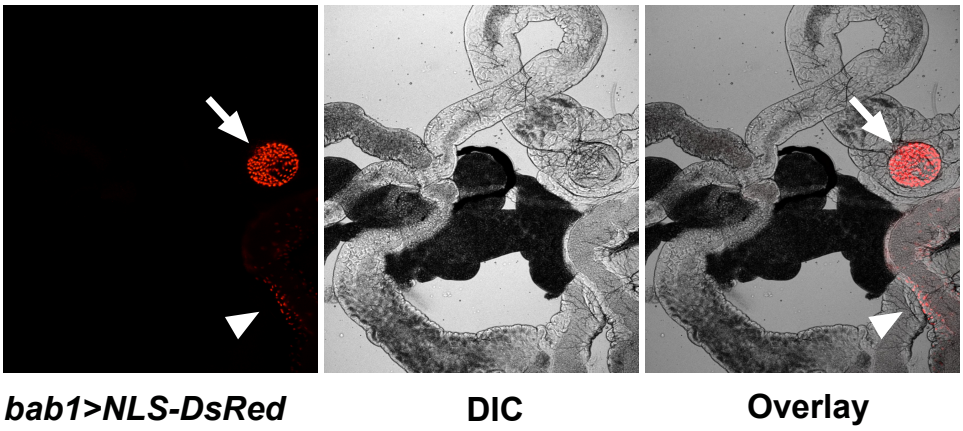


Figure 3.7 *bab1-GAL4* drives expression in the proventriculus region of the intestine

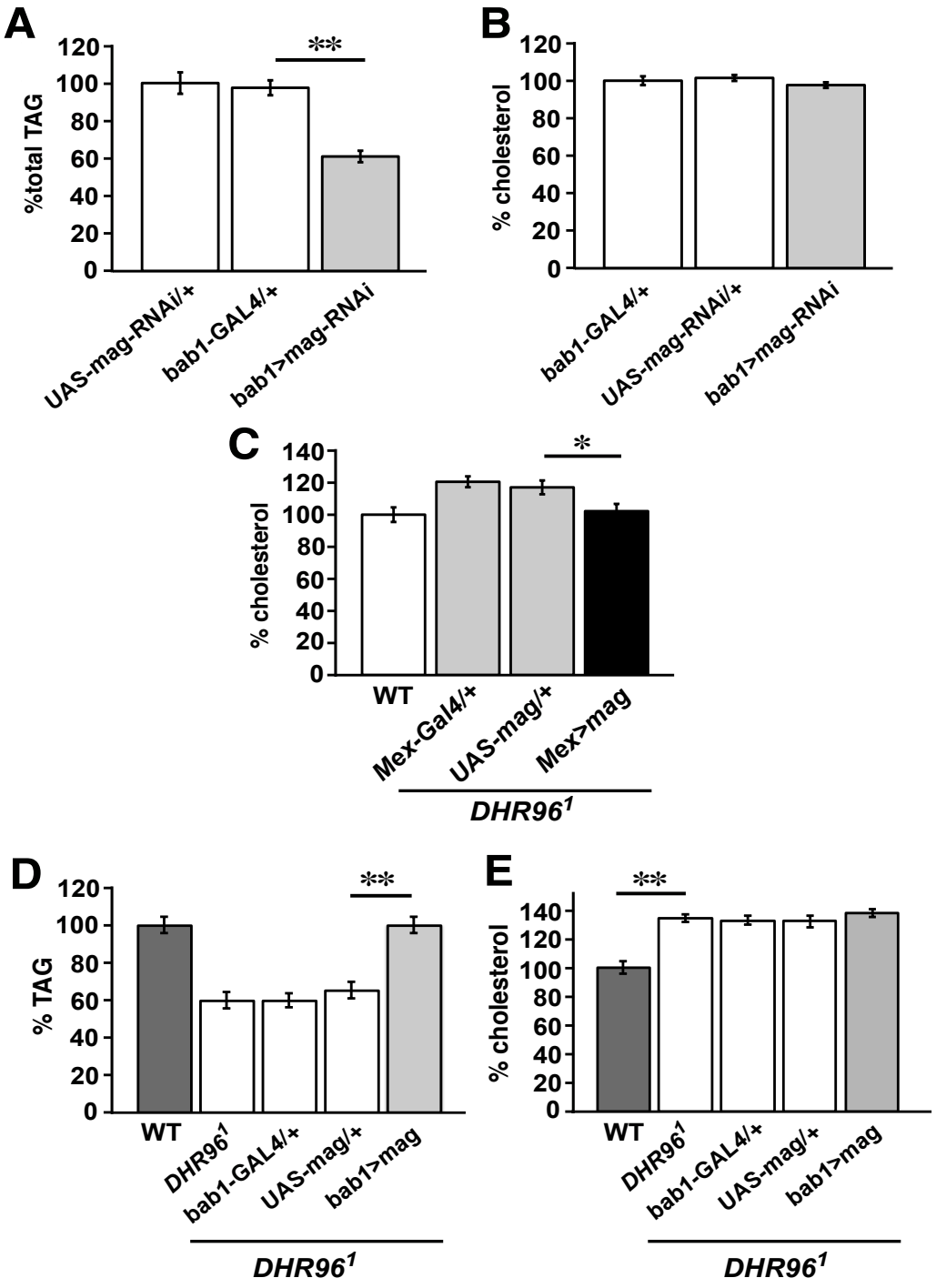
The *bab1-GAL4* transgene was used to drive the expression of *UAS-NLS-DsRed,* confirming that *bab1- GAL4* is highly expressed in the proventriculus region of the intestine (arrow), with low level expression near the midgut/hindgut junction (arrow head).

magro functions in the proventriculus to promote the breakdown of dietary TAG

The apparent vesicular trafficking of Magro protein from the proventriculus toward the body of the midgut provides a mechanism to explain its delivery into the lumen of the intestine. If this model is correct, then disrupting *magro* function specifically in the proventriculus should have an effect on TAG levels, but little or no effect on cholesterol homeostasis. To test this hypothesis, we used the *bab1-GAL4* driver, which is expressed highly in the proventriculus, with lower levels near the midgut-hindgut junction (Figure 3.7)(Cabrera et al., 2002). Using this construct to direct *magro* RNAi resulted in a significant decrease in whole animal TAG levels relative to the *bab1-GAL4/+* and *UAS-magro RNAi/+* controls (Figure 3.8A). This level of reduction is

Figure 3.8 DHR96 regulation of *magro* in distinct regions of the intestine coordinates TAG and cholesterol homeostasis

(A,B) Proventriculus-specific RNAi for *magro* results in reduced levels of TAG, but has no effect on cholesterol. *bab1-GAL4*/+ and *UAS-magro-RNAi*/+ control flies, along with *bab1-GAL4*/*UAS-magro-RNAi* (*bab1>mag-RNAi*) animals were assayed for (A) total TAG and (B) cholesterol levels. The data were normalized to protein levels and are presented relative to a wild-type level of 100%. (C) Midgut-specific expression of *magro* is sufficient to partially rescue the elevated cholesterol levels in *DHR96* mutants. Wild-type (WT) and *DHR96* mutants, carrying either the *Mex-GAL4* driver alone, *UAS-magro* transgene alone, or both *Mex-GAL4* and *UAS-magro* (*Mex>mag*) to express *magro* in the midgut, were assayed for total cholesterol levels. The data were normalized to protein levels and are presented relative to a wild-type level of 100%. (D,E) Proventriculus-specific expression of *magro* is sufficient to rescue the lean phenotype of *DHR96* mutants, but has no effect on its elevated cholesterol levels. Wild-type (WT) and *DHR96* mutants, carrying either the *bab1-GAL4* driver alone, *UAS-magro* transgene alone, or both *bab1-GAL4* and *UAS-magro* (*bab1>mag*) to express *magro* in the proventriculus, were assayed for (D) TAG and (E) cholesterol levels. The data were normalized to protein levels and are presented relative to a wild-type level of 100%. Results are compiled from six replicate samples and repeated at least three times. Error bars represent ±SEM (*$p < 0.01$ **$p < 0.0001$).


A
%total TAG
120
100
80
60
40
20
0
**
UAS-mag-RNAi/+
bab1-GAL4/+
bab1>mag-RNAi
B
% cholesterol
120
100
80
60
40
20
0
bab1-GAL4/+
UAS-mag-RNAi/+
bab1>mag-RNAi
C
% cholesterol
140
120
100
80
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40
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*
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Mex-Gal4/+
UAS-mag/+
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UAS-mag/+
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similar to that seen when *magro* function is disrupted using the pan-intestinal *Mex-GAL4* driver (Sieber and Thummel, 2009), suggesting that the TAG metabolic functions of this enzyme are restricted to the proventriculus. In contrast, proventriculus-specific *magro* RNAi has no effect on total cholesterol levels in these animals, indicating that the

DHR96 regulates major aspects of lipid metabolism through its target gene *magro*

If decreased *magro* expression is physiologically relevant to the cholesterol accumulation phenotype seen in *DHR96* mutants, then restoring *magro* function in these animals should rescue their defect in cholesterol homeostasis. To test this hypothesis, we expressed wild-type *magro* in the intestine of the *DHR96* mutant using *Mex-GAL4* and measured whole animal cholesterol levels. Relative to the *UAS-magro/+* and *Mex-GAL4/+* controls, we found that *magro* expression in the intestine of the *DHR96* mutant is sufficient to partially rescue the cholesterol accumulation phenotype seen in these animals (Figure 3.8C). The fact that this rescue is not complete is consistent with the multiple levels of cholesterol metabolism that are regulated by DHR96 (Horner et al., 2009; Bujold et al., 2010). Moreover, specific expression of *magro* in the proventriculus of *DHR96* mutants effectively rescues their lean phenotype (Figure 3.8D), but has no significant effect on the elevated levels of cholesterol in these animals (Figure 3.8E). Taken together with our other data, these results indicate that the region-specific enzymatic activities of Magro correspond to the lipid metabolic functions of DHR96. DHR96 regulation of *magro* expression in the proventriculus maintains an appropriate level of TAG lipase activity in the intestinal lumen to facilitate dietary lipid break down,

while DHR96 regulation of *magro* in the body of the intestine promotes the clearance of excess sterols.

Discussion

In addition to its essential role in nutrient digestion and absorption, recent work has identified the intestine as a key organ for the clearance of excess sterols (van der Velde et al., 2007; van der Veen et al., 2009; Lo Sasso et al., 2010). The mechanisms that underlie this transintestinal cholesterol efflux, however, remain poorly understood (van der Velde et al., 2010). This study identifies *magro* as a key factor required in the intestine to promote the clearance of excess sterols. We also show that *magro* has region-specific enzymatic activities in the intestine that correspond to the major lipid metabolic functions of its upstream regulator, the nuclear receptor DHR96. Below we discuss these activities of Magro, relate them to its mammalian homolog, LipA, and describe how studies of *magro* regulation and function can provide insights into the mechanisms by which the intestine coordinates TAG and cholesterol homeostasis.

Magro and LipA share conserved functions in maintaining lipid homeostasis

Although a number of recent genetic studies have focused on understanding the regulation of lipid metabolism in *Drosophil*a, relatively little is known about the mechanisms that control cholesterol levels in this organism, and nothing is known about how TAG and cholesterol homeostasis might be coordinated. Genetic studies of cholesterol homeostasis in *Drosophila* have focused primarily on the Niemann-Pick

(NPC) disease gene homologs, with the *Drosophila* ortholog of vertebrate *NPC1*, *npc1a*, and two of the eight fly *NPC2* homologs, *npc2a* and *npc2b*, playing important roles in intracellular cholesterol trafficking and synthesis of the steroid hormone 20-hydroxyecdysone (Fluegel et al., 2006; Huang et al., 2007). In contrast, functions for *npc1b* are restricted to the intestine, where it is essential for dietary cholesterol absorption, much like its mammalian ortholog *NPC1L1* (Voght et al., 2007). Interestingly, most of the *Drosophila* NPC genes are regulated by the DHR96 nuclear receptor, which plays a central role in cholesterol metabolism. *DHR96* mutants accumulate excess sterol, in part due to a failure to properly repress *npc1b* expression in the intestine (Horner et al., 2009). DHR96 also regulates many other genes that contribute to cholesterol uptake, trafficking, and storage, including the *LipA* homolog *magro*. The studies described here identify *DHR96* regulation of cholesterol clearance through *magro* as an important mechanism for intestinal cholesterol efflux.

Animals lacking *magro* function are lean and exhibit elevated levels of cholesterol. These roles for Magro arise from its dual enzymatic activities in the intestine, cleaving dietary TAG to facilitate fat absorption and breaking down cholesterol esters to promote cholesterol efflux. These dual functions correspond to those of its mammalian homolog LipA, and are consistent with the common fatty acid ester bond that is cleaved by the enzyme in both lipid substrates (Goldstein et al., 1975; Ameis et al., 1994). It should be noted that all of our studies of *magro* function have depended on RNAi. Although a clear prediction of this work is that *magro* null mutants would be lethal, due to an inability to break down dietary TAG, an analysis of these animals would provide valuable confirmation of our RNAi data. Numerous attempts to isolate a targeted null

allele, however, were unsuccessful, and the location of *magro* within an intron of *CG5195* complicated our ability to isolate deletion mutants by imprecise P-element excision. Future genetic studies of *magro* should provide an important means of confirming and extending the results described here with tissue-specific RNAi.

Interestingly, our studies of *magro* function in *Drosophila* reveal parallels with *LipA* function in mouse and humans. Mouse *LipA* mutants display a lack of stored fat in the form of white adipose tissue along with excess cholesterol esters (Du et al., 1998; Du et al., 2001), reflecting the major defects in *magro* mutants. Similar phenotypes are seen in patients suffering from the human lipid disorders CESD and Wolman's disease, which are caused by mutations in *LipA* (Burke and Schubert, 1972). Patients with Wolman's disease also have intestinal malabsorption, which may be related to the defects in lipid uptake that we observe in *magro* mutants. In addition to these shared phenotypes, however, mammalian *LipA* mutants display massive accumulations of lipid in the liver, spleen, and intestine – defects that are not apparent in *magro* mutant flies (Du et al., 2001). Nonetheless, the parallels between Magro and LipA function in flies and humans establish *Drosophila* as a system to further our understanding of CESD and Wolman's disease, and define the ancestral function for this class of acid lipases, demonstrating their central role in the intestine to coordinate TAG and cholesterol homeostasis.

Magro has region-specific functions in the intestine

In contrast to the detailed studies of LipA enzymatic functions, we have a relatively poor understanding of its cellular roles in the animal. Our studies of Magro have shown that the TAG lipase and cholesterol esterase activities of this enzyme act in

distinct regions of the intestine to maintain homeostasis. A genomic copy of Magro tagged with GFP reveals a low level of expression throughout the intestine, visible as punctate cytoplasmic staining in the enterocytes (Figure 3.2B). In addition, Magro-GFP is abundantly expressed in the large outer columnar cells in the anterior half of the proventriculus (Figure 3.2A). Interestingly, we also see a stream of large acidic vesicles that originate from this region of high expression and move in a posterior direction toward the lumen of the intestine (Figure 3.2 F-H, Figure 3.6). Disruption of *magro* function specifically in the proventriculus blocks its TAG lipolytic activity, but has no effect on the levels of cholesterol in these animals (Figure 3.4A,B). In contrast, *magro* RNAi throughout the intestine affects both TAG and cholesterol homeostasis, suggesting that the low level of Magro expression in the enterocytes is critical for intestinal cholesterol efflux (Figure 3.1D)(Sieber and Thummel, 2009). These region-specific functions of Magro are consistent with our dietary supplementation studies with pancreatin and Orlistat, which show that TAG lipolysis in the lumen of the intestine is required for proper fat uptake while cholesterol esterase activity in the lumen is dispensable for cholesterol homeostasis (Figure 3.3, Figure 3.7).

The apparent vesicular trafficking of Magro in the proventriculus is consistent with the cells at the anterior end of this structure (referred to as the cardia) having secretory functions. Classic studies have shown that the peritrophic matrix arises from these cells and is deposited into the lumen that lies between the outer and inner cell layers of the proventriculus (King, 1988) (Figure 3.5). The peritrophic matrix is a meshwork of chitin and glycoproteins that provides a protective lining within the gut, much like the mucosal layer of the mammalian intestine (Hegedus et al., 2009). The observation that

the Magro-GFP vesicles reside in the same region of the proventriculus as the developing peritrophic matrix suggests that they are synthesized and exported into the lumen of the gut in a similar manner. This also raises the possibility that the digestive enzymes that are embedded in the peritrophic matrix may originate from vesicular trafficking in the proventriculus. Many genes with predicted digestive functions, including glucosidases, mannosidases, and endopeptidases, are regulated by DHR96 and expressed in the intestine, like *magro* (Sieber and Thummel, 2009). Several genes that contribute to the peritrophic matrix are also regulated by DHR96. It would be interesting to determine if these proteins are synthesized and secreted in a coordinated manner by the cardia in the proventriculus.

In contrast to the role of Magro in the proventriculus to promote dietary TAG hydrolysis, this enzyme acts outside the proventriculus in the intestine to remove excess cholesterol. The expression of Magro in the enterocytes provides a possible mechanism to provide this function. We propose that *magro* acts as a cholesterol esterase in these cells, breaking down stored cholesterol to facilitate its elimination from the intestine. This model is consistent with the neutral lipid stores that are known to reside in the *Drosophila* intestine, second only to the fat body. It is also consistent with recent evidence that LipA acts as cholesterol esterase in macrophage foam cells to promote cholesterol efflux (Ouimet et al., 2011), and suggests that LipA may exert a similar role in the mammalian intestine to maintain homeostasis.

DHR96 and *magro* provide a novel mechanism for intestinal cholesterol efflux

Like DHR96, mammalian LXR nuclear receptors can control cholesterol levels through the intestine, with LXR activation in this tissue resulting in a dramatic increase in fecal sterol excretion that correlates with increased expression of the *ABCG5/ABCG8* bile acid transporter (van der Veen et al., 2009; Lo Sasso et al., 2010). This observation suggests that LXR promotes reverse cholesterol transport in this tissue, which represents the best understood mechanism for eliminating excess cholesterol from the body. Reverse cholesterol transport involves HDL-mediated transport of cholesterol from peripheral tissues to the hepatobiliary tract, leading to the removal of excess sterol by biliary excretion from the body. LXR activation of fecal sterol excretion, however, proceeds normally in *abcb4* mutant mice, which have severely impaired biliary cholesterol secretion (Kruit et al., 2005). Similarly, *abcg5*/*abcg8* double mutants, which have highly reduced biliary cholesterol excretion, have relatively normal levels of fecal sterols (Yu et al., 2002). These and other studies have challenged the importance of reverse cholesterol transport for cholesterol excretion and have led to the proposal that the intestine may play a more direct role in this process (van der Velde et al., 2010). Indeed, intestinal perfusion studies in mice have demonstrated that up to 60% of fecal sterols can be directly excreted from the proximal intestine (van der Velde et al., 2007). Taken together, these studies are shifting the focus of cholesterol efflux toward the intestine and implicate a central role for LXR in regulating this poorly understood pathway.

Our work provides a new context to understand the role of LXR receptors in intestinal physiology. We show that restoring *magro* expression in the intestine of

DHR96 mutants is sufficient to prevent the accumulation of excess sterols (Figure 3.4C). This study establishes the importance of intestinal cholesterol esterase activity in clearing excess sterol from the body and suggests that acid lipases such as LipA may function downstream from mammalian LXR to maintain cholesterol homeostasis. Although there is no direct evidence that LXR regulates LipA expression, a recent study has shown that elevated levels of oxidized LDL can repress *LipA* expression in endothelial cells, and this effect can be reversed by treatment with LXR agonists (Heltianu et al., 2011). Further studies are required to determine if the regulatory links between LXR, LipA, and cholesterol homeostasis have been conserved through evolution, and if *Drosophila* can be used as a simple model system to better define the mechanisms of transintestinal cholesterol efflux.

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CHAPTER 4

TRANSGENERATIONAL METABOLIC REPROGRAMMING MEDIATED BY *DHR96* AND THE *HP1* CHROMATIN-REMODELING COMPLEX

Introduction

The ability to sense physiological state and regulate specific changes to metabolism is central to all aspects of animal life, balancing energy and biosynthesis needs with dietary nutrient uptake, synthesis, and catabolism. Consistent with these essential functions, metabolic dysfunction has been associated with a number of different forms of human disease such as cardiovascular disease and cancer (CDC, 2011; WHO, 2006). The astonishing rate at which metabolic disorders are increasing in frequency, however, exceeds the influence of simple genetic and environmental factors alone, suggesting that other mechanisms contribute to the increased prevalence of these disorders. One such factor is the remarkable correlation between metabolic status of the parent and that of their progeny. Numerous studies have described this phenomenon of nutritional or metabolic programming, demonstrating correlations between poor nutrition in pregnant mothers and metabolic disorders in the resulting adult progeny, along with an increased incidence of cancer, heart disease, diabetes and obesity (Langley-Evans, 2009) (Warner and Ozanne, 2010). These correlations are thought to arise from poor maternal nutrition and its effect on the developing fetus. One of the most extensive studies centers on the Dutch Hunger Winter, which affected a group of individuals in the Netherlands who were born during a Nazi military embargo on food and supplies during the winter of 1944-1945 (Schulz, 2010). During this period, pregnant women were subjected to temporary but severe famine, with the offspring rapidly returning to a normal diet that extended into adulthood. Individuals born during this period frequently displayed significant increases in total cholesterol and triglycerides (Lumey et al., 2009) (de Rooij et al., 2007) (Painter et al., 2005), accompanied by an elevated incidence of obesity,

diabetes, heart disease, and cancer (Kahn et al., 2009; Park et al., 2008). These observations are similar to those seen in the offspring of rodents subjected to temporary nutrient depletion (Langley-Evans, 2009) (Burdge and Lillycrop, 2010; Burdge et al., 2008; Lillycrop, 2011; Warner and Ozanne, 2010). Taken together, these results are interpreted as maladaptation, in which a poor nutritional environment for the fetus establishes a metabolic state that is carried through into adulthood, anticipating that the poor environment will continue. If, however, the nutritional environment improves after birth, then the metabolic state of the progeny is no longer matched to the environment, leading to metabolic dysfunction.

A molecular foundation to explain this phenomenon has emerged in recent years, suggesting that chromatin regulation plays a central role in the transgenerational inheritance of metabolic state. Numerous studies in mice and rats have shown that changes in parental nutrition can have an impact on the transcription of key metabolic transcription factors that correlates with changes in epigenetic marks at the promoters of these genes (Warner and Ozanne, 2010). For example, when pregnant mice are fed a diet low in protein content, the resulting progeny display an ~10-30% reduction in DNA methylation of the glucocorticoid receptor and *PPARα* promoters in the liver as well as an increase in the expression of these factors and their target genes (Burdge et al., 2007). Other studies have reported an increased incidence of diabetes in the adult F1 generation, along with changes in histone acetylation and H3K4 and H3K9 methylation in the *GLUT4, glucocorticoid receptor,* and *Pdx1* promoters (Lillycrop et al., 2007; Park et al., 2008; Raychaudhuri et al., 2008). Links have also been made between reduced hepatic *Cyp7a1* expression, increased H3K9 methylation, and elevated cholesterol levels (Sohi et

al., 2011). Overall these studies support the model that the metabolic state of the mother establishes a specific epigenetic program in the progeny that is maintained in the subsequent generation and helps dictate metabolic state. These studies, however, are all correlative and little is known about the functional effects of these changes in chromatin state on the progeny. For example, DNA methylation is commonly associated with gene expression changes; however, it remains unclear if DNA methylation is required for changes in gene expression in response to parental diet. In addition, given the *in utero* link between fetal metabolism and the metabolic state of the mother, it remains unclear if the effects observed in these studies are the result of a transmissible change in chromatin state between parental and progeny generations or if poor fetal metabolism initiates a change in metabolic state that persists into adulthood. These questions were addressed recently in a study investigating the paternal contribution to transgenerational metabolic reprogramming (Carone et al., 2010). The offspring of male mice fed a low-protein diet display a transcriptional profile characterized by up-regulation of lipid biosynthetic pathways and down-regulation of hepatic PPARα expression. While no effects on hepatic TAG and fatty acid levels were observed, these animals displayed reduced hepatic cholesterol, similar to the rat studies. These effects were correlated with a slight ~15% increase in DNA methylation of an intergenic CpG island located 50 kb upstream from the PPARα promotor in the offspring. The role of this region in *PPARa* regulation, however, remains unclear. In addition, a recent study showed that when adult male mice are fed a chronic high fat diet, female progeny of these mice exhibit defects in glucose tolerance that correlates with a slight decrease in islet cell mass, suggesting that both

nutrient depletion as well as nutrient excess can stimulate transgenerational effects on metabolism (Ng et al., 2010).

In the course of my studies of *DHR96* and *magro*, I noticed that wild-type flies display significant changes in absolute levels of TAG between experiments, and that these changes were not always apparent in the *DHR96* mutant. A series of studies revealed that these changes in TAG levels were not due to nutritional effects on that generation of flies, but rather reflected the health and nutrition of their parents. As a result, I began experiments to determine if the transgenerational inheritance of metabolic state defined in mammals can also occur in *Drosophila*. My data indicate that when parental flies are fed a nutrient poor diet, the resulting progeny show a 2-fold increase in whole animal TAG levels. Furthermore, mutants lacking DHR96 are unable to mediate these transgenerational effects, suggesting that DHR96 is a key factor in this process. Based on mammalian studies, these transgenerational effects on metabolism are likely transduced by changes in chromatin state. Consistent with this hypothesis, animals heterozygous for a strong loss-of-function mutation in *HP1* exhibit a significantly disrupted transgenerational response. This observation provides the first genetic evidence of a functional role for chromatin state in transgenerational metabolic reprogramming. My data support the model that transgenerational changes in chromatin state adjust the metabolic state of the progeny to provide them with a physiological advantage for survival in their nutritional environment.

Materials and methods

Fly stocks

Fly stocks used in this study include: *Canton S, DHR96*^{1} (King-Jones et al., 2006), and *Su(var)205*^{5}*/CyO*(Eissenberg et al., 1992), which was a gift from Gunter Rueter.

Metabolic conditioning parameters

Our strategy for metabolic programming is depicted in Figure 4.1. To determine the effects of parental diet on the metabolic status of their progeny in *Drosophila*, I set up crosses of 20 mature male and 20 mature female flies of the same genotype. These matings were conditioned on one of three media: SD (semidefined complete media), SY (sucrose/yeast extract minimal media), or SY– (lipid depleted sucrose/yeast extract minimal media) for 7-10 days. After conditioning, these flies were transferred to SD complete media and allowed to lay progeny for 3-5 days, after which the adults were discarded. The resulting progeny were allowed to develop on the SD diet until eclosion and then collected, transferred to a new SD media vial, and aged for 7 days. Once matured, the adult males were either collected for metabolite measurements (5 males/sample) or collected to assay transcript levels (20 males/sample) by northern blot hybridization.

Metabolite measurements

Metabolite measurements were conducted by collecting newly-eclosed adult male flies and aging them 5-7 days prior to use for all experiments. TAG and cholesterol assays were conducted as described (Horner et al., 2009; Sieber and Thummel, 2009).

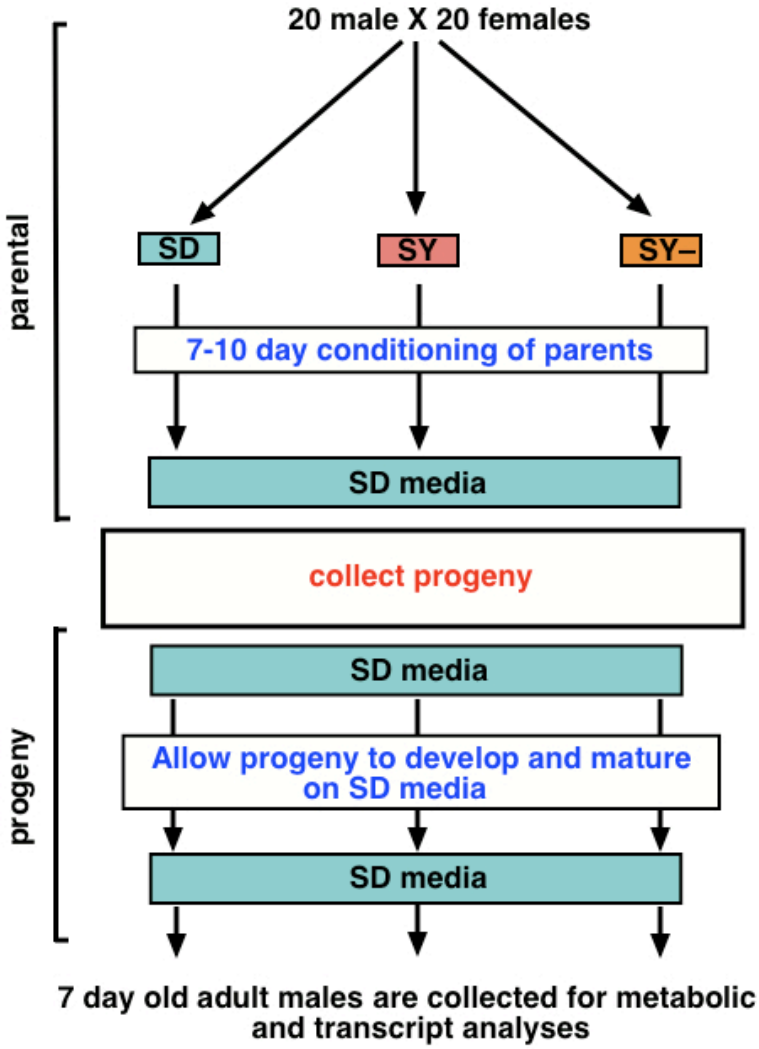

Figure 4.1 Diagram of the transgenerational metabolic conditioning regimen

This model depicts the conditioning paradigm used to determine the effects of changes in parental diet on the metabolic state of the progeny. Mature adult flies were mated and conditioned for 7-10 days on one of three media, SD, SY, or SY-lipids (SY-). These conditioned adults were then allowed to lay eggs for 3-5 days on rich SD media, after which they were discarded. The resulting progeny were allowed to develop and mature on the SD media, after which the adults were collected and assayed for metabolite and mRNA transcript levels.

Glycogen and trehalose measurements were conducted as described (Tennessen et al., 2011). All results shown are derived from 12 samples of five animals collected from each genotype under each condition, and repeated at least three times. A representative experiment is shown in each figure.

Media recipes

Semidefined media was made by combining 10 g agar (Sigma), 40 g cornmeal, 80 g of brewers yeast (Fleischmann's), 20 g of yeast extract (Sigma), 20 g of peptone (Sigma), 30 g of sucrose (Sigma), 60 g of dextrose (Sigma), 0.5 g of $MgSO_4$, and 0.5 g of $CaCl_2$ in a 2 liter flask. The volume was raised to 1 liter with ddH_2O, mixed, and microwaved until boiling. Once boiling, the mixture was microwaved for an additional 1-2 minutes to ensure that the agar had melted. The food was then allowed to cool until ~40^{o}C, then 6 mls of propionic acid and 10 mls of 10% tegosept were added. The food was mixed and dispensed into vials.

SY media was made by adding 30 g of yeast extract (Sigma), 30 g sucrose (Sigma), and 3 g of agar (Sigma) into a 1 liter flask. The volume was raised to 300 mls and microwaved until boiling. Once boiling, the mixture was microwaved an additional 1 minute to ensure the agar had melted, and allowed to cool to ~40^{o}C. Once cooled, 1.8 mls of propionic acid and 3 mls of 10% tegosept were added. The food was then mixed and dispensed into vials.

To make lipid depleted SY–, yeast extract and agar were lipid extracted with two volumes of chloroform overnight in a fume hood. The following day, the yeast extract and agar were filtered with Whatman #1 paper and extracted a second time with two

volumes of chloroform for 4 hours. After the second extraction, the yeast extract and agar were filtered with Whatman #1 paper and placed uncovered in a fume hood for 3 days to allow the chloroform to evaporate completely, before being used to make SY– media as described above for SY.

Results

Parental diet dictates changes in stored energy in the progeny

To determine if parental diet influences the metabolic state of their progeny in *Drosophila*, matings of wild-type flies were set up and conditioned for 7-10 days on either semidefined (SD) complete media, sucrose/yeast extract (SY) minimal media, or lipid depleted sucrose/yeast extract (SY–). Once conditioned, progeny from these animals were collected and allowed to develop and mature on SD complete media (Figure 4.1). I then assayed the adult males for TAG and found that, relative to the SD unconditioned controls, progeny whose parents were subjected to either the SY or SY– minimal media exhibited a significant ~2-fold increase in TAG levels (Figure 4.2A). In contrast, I observed a significant decrease in glycogen levels in these animals, suggesting that transient exposure to a minimal diet results in a shift in the forms of stored energy in their offspring, from carbohydrate to lipids (Figure 4.2B).

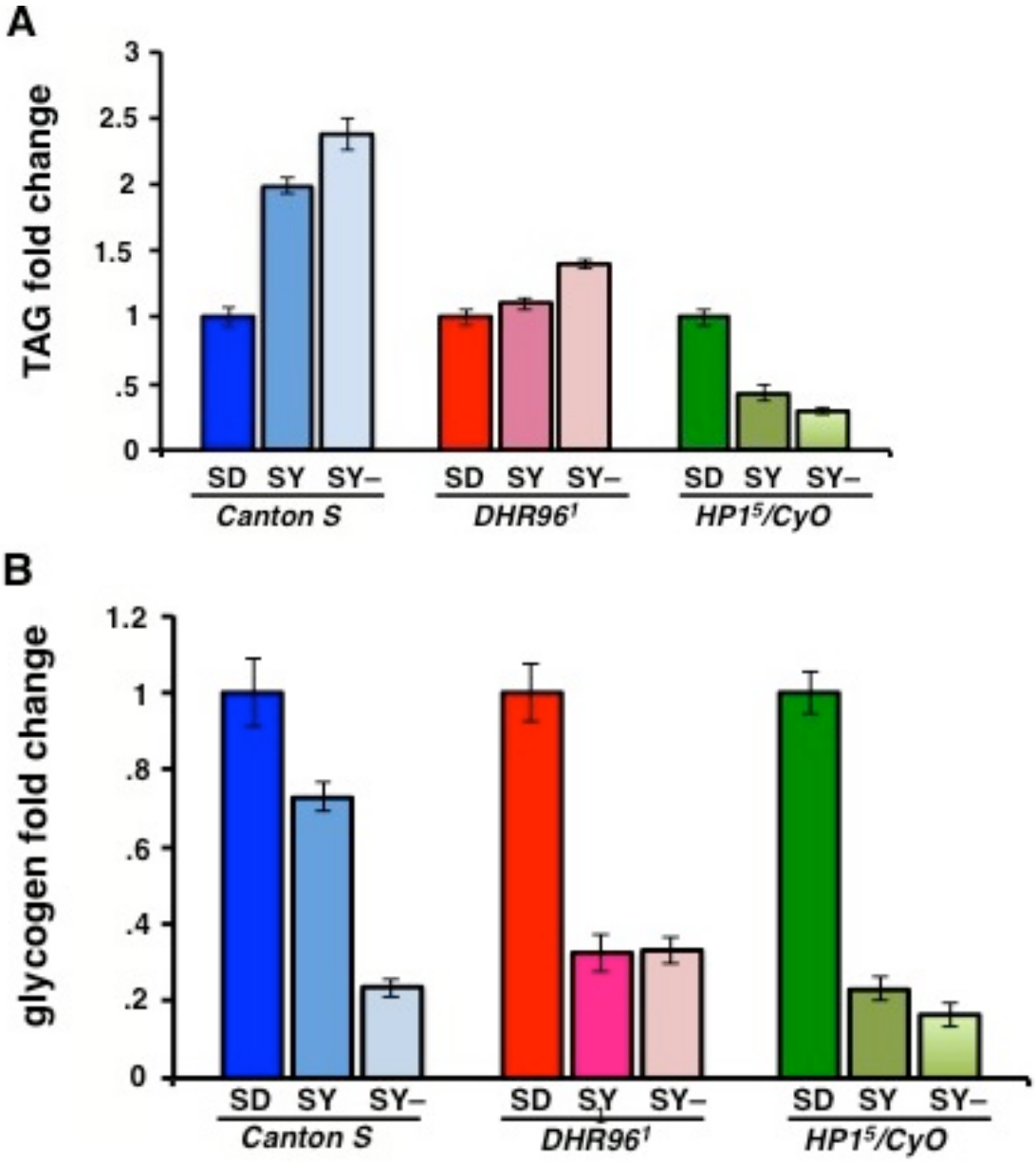

Figure 4.2 TAG and glycogen levels in the progeny of *Canton S, DHR96[1]*, and *HP1[5]/CyO* parents.

Canton S wild-type flies, *DHR96[1]* mutants, and *HP1[5]/CyO* transheterozygous animals were subjected to the conditioning regimen described in Figure 4.1. The resulting mature adult progeny were assayed for TAG (A) or glycogen levels (B). The data presented are depicted as fold change relative to the control that was maintained continuously on the SD media. Error bars indicate 1xSEM.

DHR96 and *HP1* are required to mediate the effects of parental diet on the metabolic state of their progeny

My initial studies suggested that mutants lacking *DHR96* might be resistant to changes in TAG levels in response to changes in parental diet. When *DHR96* mutant parents are subjected to our standard conditioning regimen (Figure 4.1), the resulting progeny display similar changes in glycogen levels to those seen in wild-type animals (Figure 4.2B). These mutant progeny, however, do not exhibit an increase in TAG levels when their parents are exposed to either SY or SY– relative to the SD unconditioned controls, indicating a role for *DHR96* in mediating this transgenerational response to parental diet (Figure 4.2A,B). In contrast, animals heterozygous for a loss-of-function mutation in *HP1 (Su(var)205)* display an inverted response to parental diet. When parental animals are conditioned using the previously described regimen (Figure 4.1), the *HP1* heterozygote progeny from parents subjected to the SY diets exhibit a significant ~2-3 fold decrease in whole animal TAG, in contrast to the increased TAG levels seen in wild-type flies (Figure 4.2A). This result suggests that HP1 contributes to the metabolic reprogramming of progeny in response to parental diet.

Excess cholesterol accumulation in the *DHR96* mutant is dependent on parental diet

Given that TAG and cholesterol metabolism are tightly coordinated by multiple mechanisms, I hypothesized that cholesterol homeostasis might also be influenced by parental diet. The progeny of wild-type and *HP1* heterozygous animals, however, show no significant effects of parental diet on cholesterol levels in their offspring, suggesting

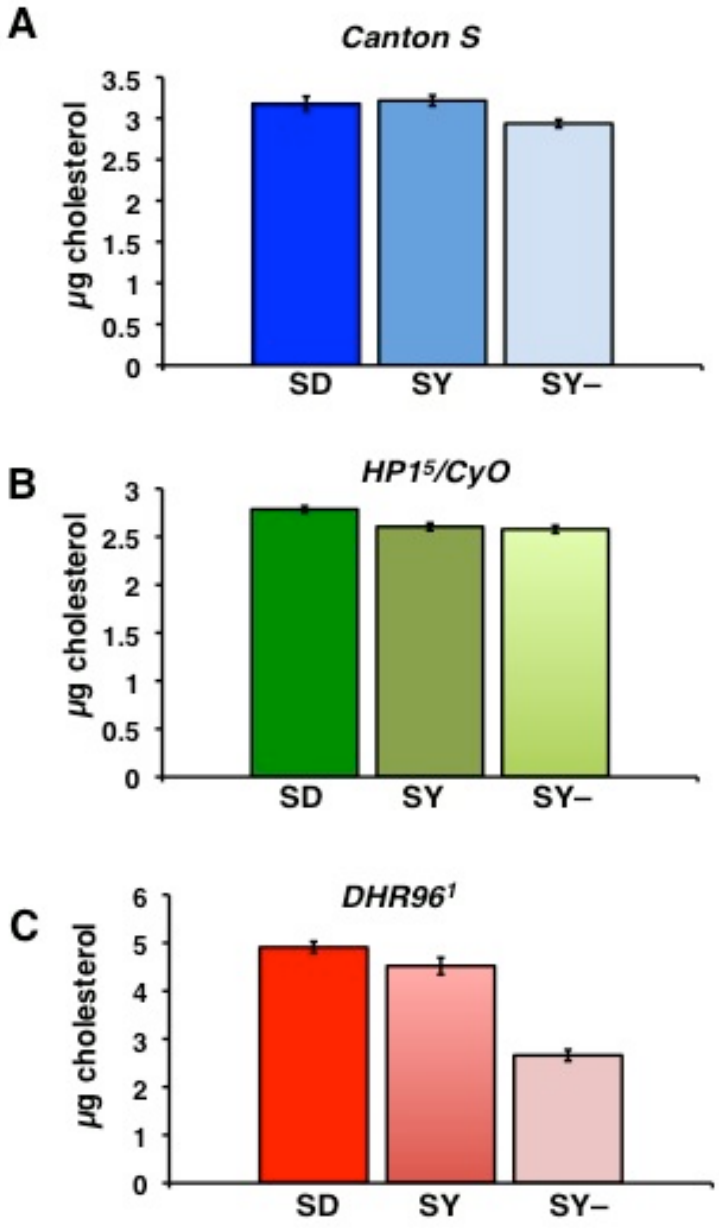

Figure 4.3 Cholesterol levels in the progeny of *Canton S, DHR96*^{1}, and *HP1*^{5}*/CyO* parents.

Canton S wild-type flies, *DHR96*^{1} mutants, and *HP1*^{5}*/CyO* transheterozygous animals were subjected to the conditioning regimen described in Figure 4.1. The resulting adult progeny were assayed for total cholesterol levels. The data presented are depicted as fold change relative to the control that was maintained continuously on the SD media. Error bars indicate 1xSEM.

that TAG metabolism is being specifically affected by parental diet independent of the mechanisms that control cholesterol levels (Figure 4.3). In contrast, when *DHR96* mutant parents are subjected to lipid depleted SY– media, the resulting progeny no longer exhibit the elevated levels of cholesterol that are normally seen in *DHR96* mutants indicating that defects in cholesterol homeostasis observed in the *DHR96* mutant are influenced by the lipid content of the parental diet. (Bujold et al., 2010) (Horner et al., 2009) (Figure 4.3).

DHR96 and *HP1* display sex-specific parental roles in the transgenerational regulation of metabolism

While my data define roles for both DHR96 and HP1 in mediating the effects of parental diet on the metabolic state of their progeny, it remains unclear whether these genes function in the parents or the progeny. I determined if DHR96 has a role in the parental generation by setting up reciprocal matings between *DHR96* mutants and wild-type *Canton S* animals and conditioning these crosses as described (Figure 4.1). Interestingly, while heterozyogous progeny from *DHR96* mutant mothers display a wild-type response to parental diet, heterozyogous progeny from *DHR96* mutant fathers no longer display this phenotype, but rather appear to recapitulate my previous result with *DHR96* homozygous mutants (Figure 4.4). These data suggest that *DHR96* functions primarily in the male parents to facilitate its role in storing TAG in response to parental diet. Conversely, when I set up reciprocal crosses between $HP1^{5}/CyO$ and *Canton S* animals and conducted the same experiment, I found that the decreased levels of TAG

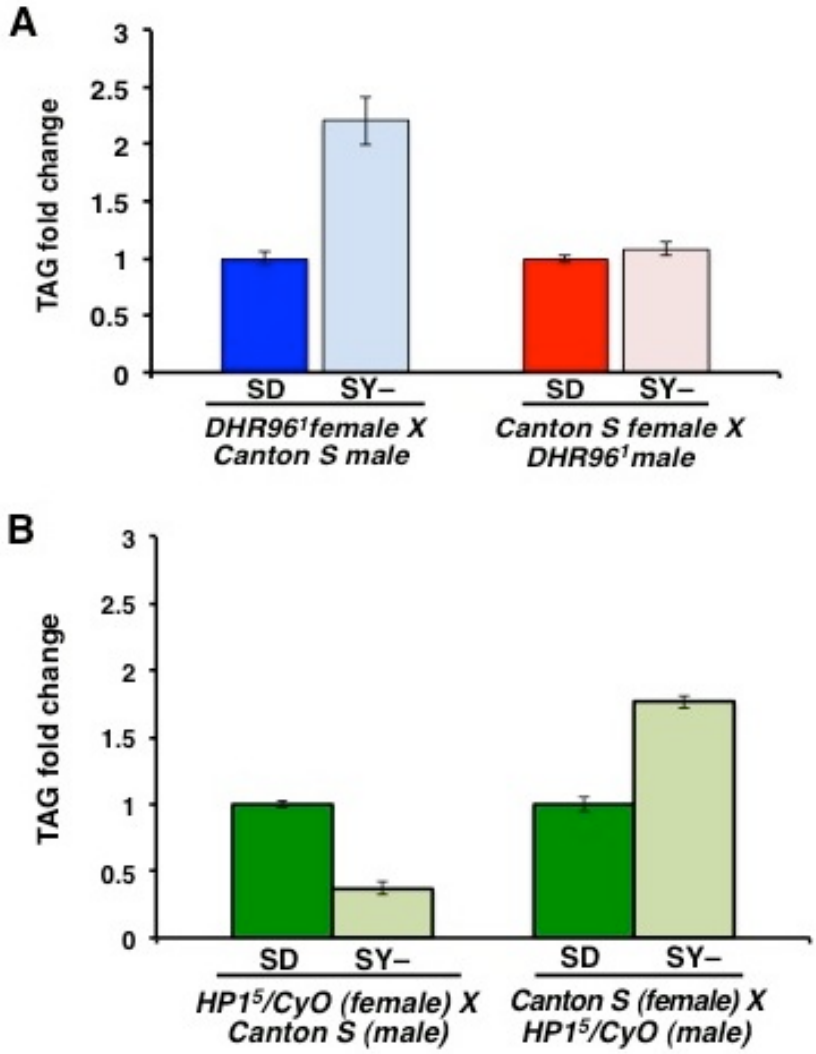

Figure 4.4 Parent-specific effects of *DHR96* and *HP1* in the transgenerational metabolic response.

(A) Reciprocal crosses were set up between *Canton S* and *DHR96*^{1} mutants. These matings were subjected to the same conditioning regimen depicted in Figure 4.1. The resulting mature adult progeny were assayed for total TAG levels. (B) The same was done using reciprocal crosses between *Canton S* and *HP1*^{5}*/CyO* animals, measuring TAG levels in the progeny. The data presented are depicted as fold change relative to the control that was maintained continuously on the SD media. Error bars indicate 1xSEM.

seen in my original studies of *HP1* heterozygotes was recapitulated with $HP1^{5}/CyO$ mothers, suggesting that *HP1* is required in the parental female for a wild-type response to parental diet (Figure 4.4). These haplo-insufficient effects are consistent with the previously described dose-dependent effects of *HP1* on position effect variegation. Taken together these results suggest that, in response to metabolic status, *DHR96* and *HP1* mediate signals through both parents to control the transgenerational metabolic state of their progeny.

Parental diet influences metabolic gene expression in their progeny

As a means to identify a molecular mechanism for these transgenerational effects on metabolism, I conditioned *Canton S, DHR96* mutant, and $HP1^{5}/CyO$ animals and collected RNA from the resulting adult progeny. Transcript levels of a number of known metabolic genes were examined by northern blot hybridization, focusing on genes regulated by *DHR96* (Figure 4.5A). Interestingly, several of these genes significantly change their expression in response to parental diet, including *magro* and two genes that encode predicted glycosyl hydrolase enzymes that contribute to carbohydrate break down: *CG9466* and *CG9468*. *DHR96* expression is also modulated by parental diet, suggesting that, in addition to its role in the parental male, mis-regulation of *DHR96* may also impact the metabolic status of the progeny (Figure 4.5B). In contrast, the transcripts of other metabolic transcriptional regulators such as *Sir2* and *HNF4* do not appear to be affected by parental diet, suggesting that DHR96 has a specific role in this process. Furthermore, the transcript levels of these genes do not change in response to parental diet in the *DHR96* mutant, consistent with the inability of the mutant to respond to

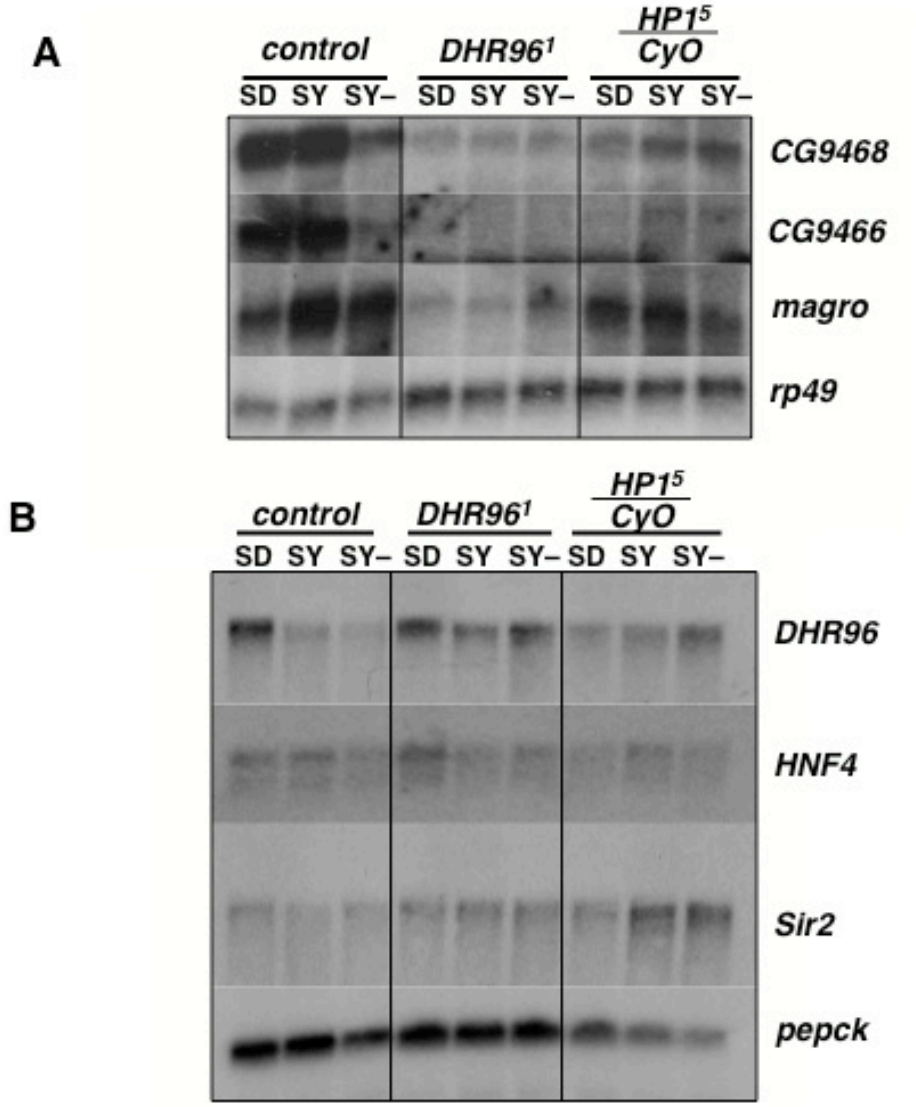

Figure 4.5 Transcriptional regulation of metabolic gene expression is dependent on parental diet.

(A) *Canton S* wild-type flies, *DHR96*[1] mutants, and *HP1*[5]*/CyO* transheterozygous animals were subjected to the conditioning regimen described in Figure 4.1. The resulting adult progeny were assayed by northern blot hybridization to detect the expression of *CG9468, CG9466,* and *magro* mRNA. Hybridization to detect *rp49* mRNA is shown as a loading control. (B) The expression of three known transcriptional regulators of metabolism, *DHR96, HNF4, and Sir2*, was assayed in these same RNA samples. Hybridization to detect *pepck* mRNA is shown as a loading control.

parental diet. In contrast, *HP1*^{5}*/CyO* animals display a disrupted, in some cases completely reversed, transcriptional response to parental diet, consistent with the transgenerational metabolic defects I observe in these animals.

Parental diet influences the transcriptional regulation of metabolic genes in larvae

While my data shows that parental diet influences both the metabolic state and transcription of metabolic genes in the resulting adult progeny, it remains unclear whether these effects manifest in the adult or arise earlier in development and persist throughout life. To distinguish between these possibilities, I conditioned *Canton S* and *DHR96* mutants using the same regimen as described above (Figure 4.1), but this time I collected early wandering third instar larvae for northern blot hybridization analysis. I examined the expression of a number of metabolic genes, focusing on genes known to be regulated by DHR96, including: a *Drosophila* NPC2 homolog *CG3934*, the intestinal endopeptidase Jon99Fi, *CG9466* and *CG9468*, which encode predicted glycosyl hydrolase enzymes, and *magro* (Figure 4.6). As I saw previously, all of these genes, with the exception of *CG3934*, exhibit some form of transcriptional regulation in response to changing parental diet, suggesting that the effects I observed on metabolic status likely arise early in development and persist throughout the life of the animal (Figure 4.6). Furthermore, these transcriptional changes in response to parental diet appear disrupted in the *DHR96* mutant (Figure 4.6). My studies, however, are limited to only a few genes and the effects of changes in parental diet on the metabolic status of larvae have yet to be characterized.

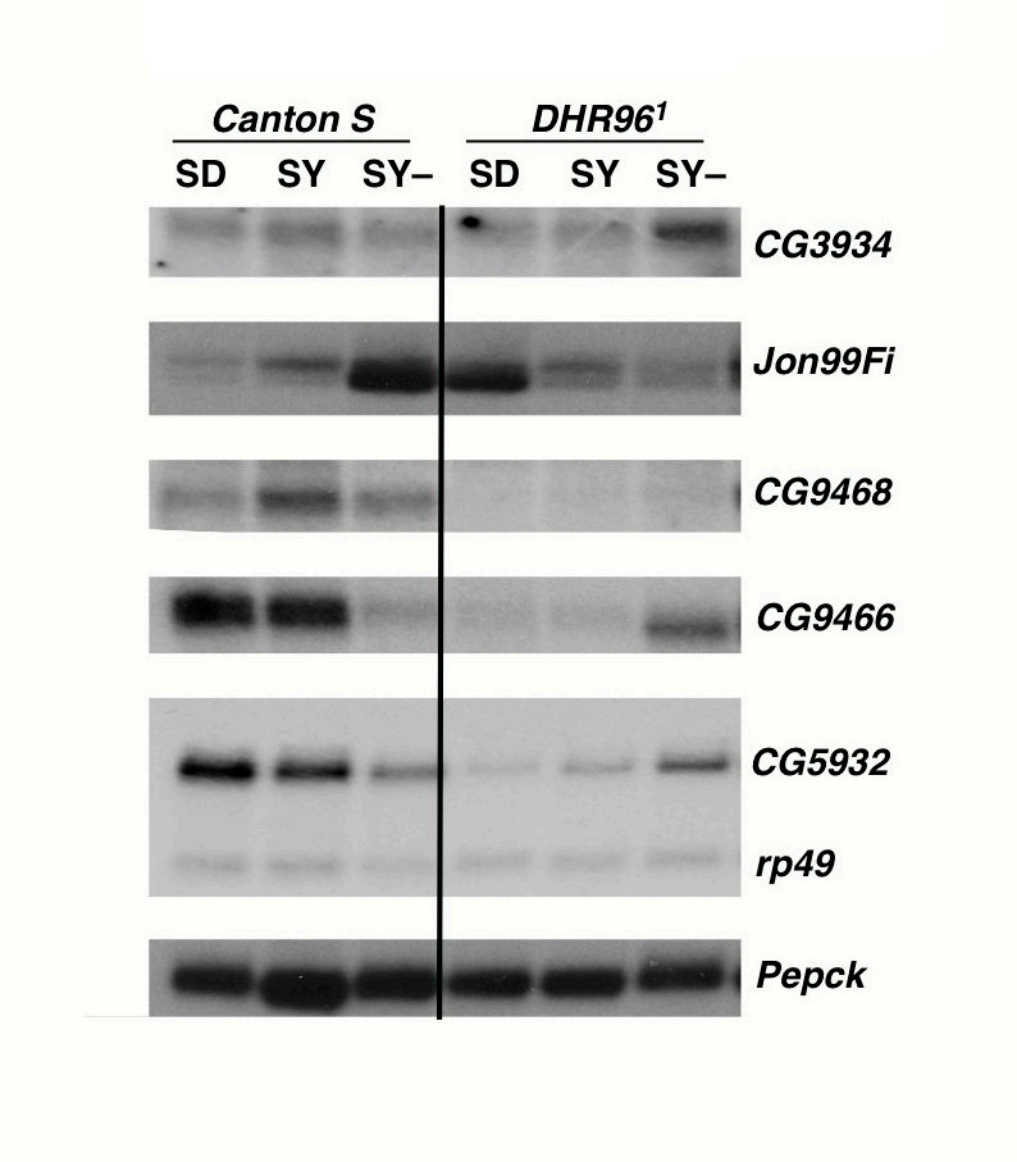

Figure 4.6 The expression of metabolic genes in larvae is dependent on parental diet.

Canton S wild-type flies and *DHR96*^{1} mutants were subjected to the conditioning regimen described in Figure 4.1. The resulting late third instar larval progeny were assayed by northern blot hybridization to detect the expression of *Jon99Fi, CG3934, CG9466, CG9468,* and *magro* mRNA. Hybridization to detect *rp49* and *pepck* mRNA is shown as a loading control.

Discussion

While studies of transgenerational effects on progeny metabolism have identified correlations between change in gene expression, chromatin modifications, and physiological effects, these studied are complicated by the limitation of the system. For example, due to the *in utero* metabolism link between the fetus and mother, understanding whether the transgenerational metabolic effects observed are the result of a transmitted signal from mother to progeny or they arise from disrupting the metabolic status in the womb itself remain unclear. *Drosophila*, however, does not maintain an *in utero* link during embryogenesis. Furthermore, the fly has multiple checkpoints that monitor metabolic state in the germline, if metabolic state is disrupted the germline stem cells stop dividing and the developing oocytes arrest and undergo apoptosis (Buszczak et al., 2002; Drummond-Barbosa and Spradling, 2001; McLeod et al., 2010). As a result the fly provides an ideal model for molecular and genetic characterization of the mechanisms that mediate these transgenerational effects on metabolism.

My initial studies in flies have established an experimental paradigm that allows us to begin to identify and characterize the mechanisms involved in the transgenerational regulation of progeny metabolism by parental diet. These studies show when parental flies are fed a nutrient poor diet for 7-10 days the resulting progeny, despite being fed a standard media throughout their life, exhibit a significant increase in TAG and a significant decrease in whole animal glycogen levels. These data indicate that when the parental generation is grown on a poor nutrient diet the progeny exhibit a redistribution of stored energy, favoring high fat storage at the expense of stored carbohydrates. Furthermore, these effects are consistent with studies in mice and humans that show

similar effects of parental diet ion the metabolic status of the progeny (Carone et al., 2010; Langley-Evans, 2009; Ng et al., 2010; Warner and Ozanne, 2010). These data also suggest that parental metabolic/ nutritional status induces this redistribution in stored energy to give the progeny a physiological advantage to survive in the environment they are born into. However, this hypothesis has not been tested.

Previous work in mammals has identified correlations between these transgenerational metabolic responses and changes in DNA methylation. However, the data supporting this hypothesis are only correlative. For example, multiple studies have shown that PPARα expression changes in response to parental diet. However a recent mouse study has shown that DNA methylation state change only about 15% at a CpG island thought to be a PPARα enhancer region, suggesting that DNA methylation is not sufficient to explain the changes in gene expression observed in these mice. In addition, studies of the human *IGF2* loci, one of the best-characterized epigenetically regulated loci in mammals, show only a small 5-10% change CpG island methylation supporting the hypothesis that other processes such as histone tail modification may play important roles in this transgenerational metabolic response. While DNA methylation is absent in flies after the first few hours of embryogenesis, flies do provide an excellent means to address the regulation of chromatin state. In fact, many of the best characterized regulators of chromatin state, such as the HP1 and polycomb complexes, were first identified and characterize in *Drosophila* (Eissenberg et al., 1990; Lewis, 1978; Tschiersch et al., 1994). My data, for the first time, provide functional evidence for the role of chromatin state in the transgenerational regulation of metabolism in response to parental diet. When $HP1^{5}/CYO$ parental animals are fed a nutrient poor diet the resulting

heterozygous progeny no longer display TAG accumulation, but instead display a decrease in TAG levels when the parents were fed a nutrient poor diet. These data are consistent with a role in the parent for chromatin regulation mediating transgenerational effects on metabolism and suggests that the HP1 chromatin-remodeling complex may be involved in this process.

Although previous studies in mammals have implicated transcriptional regulation of nuclear receptors, such as the glucocorticoid receptor and *PPARα,* as a possible mechanism for mediating changes to metabolism in the progeny in response to parental diet, there are no genetic studies that support this hypothesis. Although the *Drosophila* genome does not encode clear orthologs for these receptors, my studies have shown that the expression of the *Drosophila* LXR homolog, DHR96, is modulated in response to parental diet. Furthermore, when *DHR96* mutant parents are fed a poor nutrient diet, the resulting progeny exhibit no changes in TAG levels in response to parental diet despite the progeny being grown on a complete media suggesting that DHR96 is an essential factor required to mediate this response. This defect in transgenerational TAG response correlates with the inability of DHR96 target genes to change their expression in response to parental diet in the *DHR96* mutant. However, the functional targets for DHR96 in mediating transgenerational metabolic effects have not been identified. Taken together these data indicate that, in conjunction with being regulated on the transcriptional level in response to parental diet, nuclear receptors like DHR96 can function in the parental generation to mediate this transgenerational metabolic response. While my work defines a role for DHR96 in the parent to facilitate this transgenerational metabolic response to parental diet, my data also suggest that DHR96 may function in the progeny to play a role

in this response. However, this hypothesis has not been tested. Previous studies have shown that nuclear receptors function to regulate transcription, in part, through the recruitment of well characterized chromatin remodeling complexes such as: SMRT, NCoR, SWI/SNF, GCN5/PCAF, CBP, SMCC, and SRC-1/3 (Chen and Evans, 1995; Dilworth and Chambon, 2001; Glass and Rosenfeld, 2000; Horlein et al., 1995). This ability in conjunction with the nuclear receptor's capability to sense metabolic status suggests nuclear receptors may play a key role in this mediating a metabolic responsive signal between generations. Nuclear receptors may function to sense parental metabolic status and facilitating changes in chromatin state, through recruitment of known chromatin regulating complexes, which can be transmitted into the progeny. These roles for DHR96 in chromatin regulation, however, have not yet been investigated.

My studies of HP1 and DHR96 define roles for both female and male parents in facilitating these transgenerational effects on metabolism. Whereas DHR96 functions in the male parent to facilitate its effects on this transgenerational metabolic response, my data indicate that the HP1 complex functions in female parents to facilitate its role in the process. Taken together these data indicate that distinct molecular mechanism function in male and female parents to mediated these effects on progeny metabolism in response to parental diet. Further studies characterizing the physiological changes and molecular mechanism affected in both male and female parents should shed light on the precise mechanisms function in both parents that are required for these effects on progeny metabolism in response to parental diet.

Future Directions

These studies establish a framework for the physiological, genetic, and molecular dissection of the mechanisms that transduce this transgenerational metabolic response to parental diet. Future studies will define the physiological and molecular effects these diets have on the parents themselves and how those effects are transmitted to the progeny. Genome wide RNA sequencing analysis will be conducted to determine the specific aspects of metabolism that are altered in the progeny in response to parental diet. To understand the role DHR96 and HP1 in this transgenerational metabolic response to parental diet, studies to assess chromatin state in control, DHR96 mutants, and HP1 heterozygotes will be conducted to define the precise chromatin modifications that function to transduce these signals between generations in response to parental diet. Overall these initial studies have identified the first factors, DHR96 and HP1, that function in the transgenerational regulation of progeny metabolism and provide a framework with which to further elucidate the molecular mechanisms involved in transducing these transgenerational signals.

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CHAPTER 5

CONCLUSIONS

Many nuclear receptors in *Drosophila* have been characterized for their critical roles in development. While these studies have defined the essential functions of these receptors, surprisingly little is known about their nonessential functions in processes like metabolic regulation, which may have significant implications for human disease. My studies define a role for the *Drosophila* DHR96 nuclear receptor in coordinating TAG and cholesterol homeostasis, similar to the mammalian LXR receptors. My work indicates that DHR96 controls the breakdown of dietary TAG through direct regulation of the intestinal lipase Magro. Previous studies have shown that DHR96 functions to sense cholesterol levels and maintain cholesterol homeostasis (Bujold et al., 2010; Horner et al., 2009). Interestingly, in conjunction with its role in breaking down dietary TAG, DHR96 functions, in part, through *magro* to maintain cholesterol homeostasis. Magro exhibits a high degree of both TAG lipase and cholesterol esterase activity. While *magro* functions in the intestinal lumen to break down dietary TAG, my work indicates that *magro* acts within the cells of the intestine to breakdown stored cholesterol esters as a means to clear excess sterols. Overall these studies identify the intestine as a key organ that coordinates TAG and cholesterol homeostasis in the fly and define roles for *DHR96* and *magro* in both of these processes.

magro functions downstream of *DHR96* to coordinate TAG and cholesterol homeostasis

While numerous studies have identified transcription factors such as SREBP and LXRs that coordinately regulate TAG and cholesterol homeostasis, it remains unclear whether single direct targets of these transcription factors function in both pathways. Interestingly, human genetic studies of patients suffering from cholesterol ester storage disease, a condition where affected individuals display severely depleted white adipose tissue and increased circulating and stored hepatic cholesterol, identified mutations in the *magro* homolog, *LipA*, as a primary causal factor (Burke and Schubert, 1972; Goldstein et al., 1975). Biochemical studies have shown that LipA directly regulates fat and cholesterol metabolism through its ability to breakdown triglycerides and stored cholesterol esters (Ameis et al., 1994; Goldstein et al., 1975). This cholesterol esterase activity of LipA is required to promote the clearance of excess sterols by ABCA1 dependent cholesterol efflux (Ouimet et al., 2011). My studies of the *DHR96* target gene, *magro*, define a similar role for this enzyme in the intestine of the fly to prevent the accumulation of excess sterols. However, the precise mechanism of sterol excretion remains unknown. Conversely, although the mechanism by which LipA regulates TAG homeostasis remains unclear, my studies of Magro suggest that this family of enzymes maintain TAG homeostasis by promoting the breakdown of dietary TAG. Consistent with a role in in the digestion of dietary lipids, mouse LipA is expressed at high levels in the intestinal villi and the exocrine pancreas. Tissue specific functions for LipA in the liver, intestine, and pancreas, however, have not yet been investigated (Du et al., 1996). Overall

my studies suggest that individual target genes, such as *magro*, can act downstream of nuclear receptors to coordinate multiple aspects of lipid homeostasis.

DHR96 and *magro* are required for intestinal cholesterol homeostasis

Genetic and molecular studies in mice have focused on the liver as an important organ for maintaining cholesterol homeostasis by promoting the clearance of excess sterols through reverse cholesterol transport (Ameis et al., 1994; Du et al., 1998; Peet et al., 1998; Repa et al., 2000). In contrast, recent work has shifted attention toward a key role for the intestine in this process (Lo Sasso et al., 2010; van der Velde et al., 2007). Intestinal profusion experiments have defined regions of the intestine that are responsible for up to 60% of the sterols excreted into feces. Interestingly, LXR activation has been shown to increase the rate of this transintestinal cholesterol excretion. However, the molecular mechanism for how cholesterol is excreted from the intestine has not been identified (Lo Sasso et al., 2010; van der Veen et al., 2009).

Consistent with these findings, our studies of DHR96 have identified a similar conserved role for this receptor in the intestine to prevent excess sterol accumulation (Bujold et al., 2010; Horner et al., 2009). These data also support mammalian data that implicate the intestine as a key organ for the excretion of excess sterols. Interestingly, my data indicate that, in addition to its established role in the breakdown of dietary fat, *magro* acts downstream of *DHR96* to prevent the accumulation of excess sterols. *Magro* appears to execute this dual activity through region-specific functions in the intestine – acting in the proventriculus to promote the breakdown of dietary TAG, and likely acting in the enterocytes to promote the breakdown of excess cholesterol esters. My studies with

DHR96 and *magro* identify the first factors in the intestine that are required for the clearance of excess sterols in the fly. These studies also suggest that *LXR* regulation of *LipA* in the intestine is an evolutionarily conserved mechanism that maintains cholesterol homeostasis.

Does DHR96 function as the *Drosophila* LXR?

Studies in mammals have shown that the mouse LXR nuclear receptors directly sense oxysterol levels and coordinate both TAG and cholesterol homeostasis (Janowski et al., 1996; Kalaany and Mangelsdorf, 2006). LXR regulates cholesterol homeostasis by controlling the clearance of excess liver sterols by promoting bile acid production and excretion (Peet et al., 1998; Repa et al., 2002; Yu et al., 2002a; Yu et al., 2002b). In addition, LXRs control TAG homeostasis through regulation of SREBP-1c, an essential transcriptional regulator that regulates multiple enzymes in the fatty acid and triglyceride biosynthetic pathways. While these studies begin to address the roles for LXRs in lipid metabolism, much remains to be learned about how other direct targets of LXRs contribute to these pathways.

The *Drosophila* genome encodes two nuclear receptors that show significant homology to LXR, EcR and DHR96. Previous studies of the *Drosophila EcR* have shown that this receptor functions primarily as an essential signal that drives developmental progression in flies, and does not fulfill the metabolic role of LXRs (Karim and Thummel, 1992; Koelle et al., 1991; Yao et al., 1993). In contrast, much like LXRs, DHR96 binds sterols and regulates both TAG and cholesterol homeostasis (Bujold et al., 2010; Horner et al., 2009). While DHR96 does promote TAG accumulation, it does not appear to do so

through regulation of SREBP, as in mammals. Studies in the second chapter of this dissertation indicate that DHR96 maintains TAG homeostasis by promoting the breakdown of dietary TAG through regulation of the intestinal lipase Magro. While it remains unclear whether LXRs can affect TAG metabolism independently of the SREBP-1c pathway, recent studies have begun to define roles for this receptor in the mouse intestine, raising the possibility that LXRs could direct the digestion of dietary TAG similar to DHR96 (Kruit et al., 2005; Lo Sasso et al., 2010; van der Veen et al., 2009).

In mammals, hepatic bile acid production and excretion are thought to be the primary means of sterol clearance (Yu et al., 2002b). LXRs mediate this process by inducing the expression of the bile acid biosynthetic enzyme, Cyp7A, and promoting bile acid excretion through regulation of the sterol transporters ABCG5/ABCG8 (Kalaany and Mangelsdorf, 2006; Peet et al., 1998; Repa et al., 2002). Interestingly, recent data have shown that mutants lacking *ABCG5/ABCG8* show a relatively small defect in fecal sterol excretion, suggesting that LXRs have additional targets that are important for the excretion of excess sterols (Yu et al., 2002a). Consistent with these data, my studies indicate that DHR96 functions through *magro* to regulate the breakdown of stored cholesterol esters and promote the clearance of excess sterols. These data, in conjunction with recent evidence that LXRs can act in the intestine to promote the clearance of excess sterols in mice (Kruit et al., 2005; Lo Sasso et al., 2010; van der Veen et al., 2009), suggest that LXRs exert this effect through the mammalian homolog of *magro, LipA*. Although there is no direct evidence that LXR regulates *LipA* expression, a recent study has shown that elevated levels of oxidized LDL can repress *LipA* expression in endothelial cells, and this effect can be reversed by treatment with LXR agonists

(Heltianu et al., 2011). Further studies are required to determine if the regulatory links between LXR, LipA, and cholesterol homeostasis have been conserved through evolution, and if *Drosophila* can be used as a simple model system to better define the mechanisms of transintestinal cholesterol efflux.

Parental diet influences the metabolic state of the progeny

The ability to sense metabolic status and regulate specific aspects of metabolism is central to human health and disease. Interestingly, numerous studies in rodents and humans have suggested that the metabolic status of the parental generation can have a profound effect on their adult progeny (Langley-Evans, 2009; Warner and Ozanne, 2010). These studies have shown that conditions of nutrient depletion or excess can lead to significant defects in the health of their progeny, along with changes in metabolic gene expression (Carone et al., 2010; Langley-Evans, 2009; Ng et al., 2010; Warner and Ozanne, 2010). Recent studies to determine a biological role for the transgenerational regulation of metabolism have suggested that a signal is transmitted to the progeny in response to parental metabolic status, establishing a state that allows the progeny to adapt to the predicted nutritional environment. These changes in progeny metabolic status have been correlated with changes in DNA methylation and chromatin, suggesting that these transgenerational effects on progeny metabolism arise from changes in chromatin state. Due to the long generation time and restrictions on modulating diets in mammalian systems, it has been difficult to test this hypothesis in mammals.

During my studies of *Drosophila* lipid metabolism, I observed changes in TAG levels in wild-type animals that appeared to reflect the health and nutrient status of the

parental flies. In subsequent experiments, I was able to show that the metabolic status of progeny flies is heavily influenced by parental diet. When wild-type parental animals are exposed to a poor diet, their resulting progeny, despite being fed a complete media throughout their development, accumulate excess TAG. In contrast, glycogen levels drop dramatically in these animals. These data suggest that there is a shift in energy storage that favors storing higher levels of lipids at the expense of stored carbohydrates. These observations are consistent with the hypothesis that the transgenerational regulation of metabolism provides an adaptive advantage. When the diets of the parents and progeny, however, are mismatched, the offspring are improperly programmed and display metabolic dysfunction. While previous studies in mammals have not tested whether transgenerational metabolic regulation provides a adaptive advantage, my work in *Drosophila* allows me to test this hypothesis directly. In conjunction with the effects on metabolite levels, I have shown that the expression of a number of predicted metabolic genes is dependent on parental diet, suggesting that transcriptional regulation plays a key role in mediating these transgenerational effects. These studies establish a new genetic system for characterizing this process as well as a framework for more in-depth molecular definition of the mechanisms that facilitate the transgenerational effects on metabolism.

HP1 is required to mediate transgenerational metabolic effects on the progeny

Studies in mice and humans have suggested that the metabolic status of the parent induces epigenetic changes that are transmitted to the next generation to help them adapt

to their nutritional environment (Heijmans et al., 2008; Lillycrop, 2011; Park et al., 2008; Warner and Ozanne, 2010). For example, a recent study has demonstrated changes in PPARα expression levels in response to parental diet. The prevailing model is that chromatin state is altered in the germline in response to parental metabolic status and the resulting changes in chromatin state are transmitted to the progeny where they influence metabolic gene expression. These studies, however, have only provided correlations between effects on metabolic gene expression in response to parental diet and alterations in chromatin state. As a result, the precise mechanisms that underlie this regulation remain unclear.

Consistent with these studies in mammalian systems, I discovered that animals heterozygous for a loss-of-function mutation in the chromatin complex member HP1 no longer display an accumulation of TAG in their progeny in response to parental diet. These data suggest that HP1 plays a key role in this process and provide the first genetic evidence for a role for chromatin state in transducing the transgenerational regulation of metabolism. While my work supports the chromatin model of transgenerational metabolic regulation, it remains unclear which chromatin modifications are involved in establishing this state. Given the links between HP1 and H3K9 histone tail modifications, my data suggests that the balance of H3K9 methylation and H3K9 acetylation may influence transgenerational metabolic regulation. This has, however, not yet been tested on a molecular level. Overall, these studies establish a foundation to undertake more comprehensive genetic, molecular, and biochemical studies to fully elucidate the effects of chromatin state on this process.

DHR96 is required for the transgenerational regulation of progeny metabolic state

While studies in rodents and humans have implicated transcriptional regulation of nuclear receptors, such as the glucocorticoid receptor and *PPARα,* as controlling changes in progeny metabolism in response to parental diet, there are no genetic studies that support this hypothesis. Although the *Drosophila* genome does not encode clear orthologs for these receptors, my studies have shown that the expression of the *Drosophila* LXR homolog, DHR96, is modulated in response to parental diet. In addition, when *DHR96* mutant parents are fed a nutrient poor diet, the resulting progeny no longer exhibit TAG accumulation in response to changes in parental diet. This correlates with the misregulation of key DHR96 target genes. These studies define a critical role for DHR96 in regulating the transgenerational inheritance of metabolic state. The exact molecular mechanisms, however, require further study. In particular, several studies have shown that nuclear receptors mediate their effects on target gene expression through interactions with several well-established chromatin remodelers, suggesting that DHR96 may provide a means of sensing metabolic status in the parental generation and directing changes in chromatin state that can be transmitted to the progeny. Taken together, my studies provide a new foundation to better understand the role of nuclear receptors in transgenerational metabolic regulation and to determine if nuclear receptors provide a means of coupling metabolic status with specific chromatin modifications across generations.

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